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Modulators and Effectors of Inositol Hexakisphosphate Activity in Prostate Cancer Cells: From Clinical Prognosis to Enhanced Therapeutics

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Modulators and Effectors of Inositol Hexakisphosphate Activity in Prostate Cancer Cells: From Clinical Prognosis to Enhanced Therapeutics

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RÉSUMÉ

Chez l'homme, le cancer de la prostate (CaP) est le cancer le plus fréquemment diagnostiqué en Amérique du Nord. Lorsque localisé à la prostate, la chirurgie et la radiothérapie sont souvent efficaces bien qu'une proportion significative des patients subissent une rechute de la maladie (aussi connue comme récurrence biochimique ou RCB). Le traitement principal pour le CaP avancé ou récurent est la thérapie par privation d'androgènes (TPA), qui vise à diminuer l'activité transcriptionelle du récepteur aux androgènes (AR). Bien que la TPA puisse prolonger la vie des patients, un CaP dit androgéno-indépendant (AI) se développe éventuellement, pour lequel les traitements courants sont essentiellement de nature palliative. Plusieurs études indiquent qu'il existe plusieurs mécanismes AR-dépendants et AR-indépendants permettant aux cellules du CaP à résister à la TPA qui pourraient aussi influencer la réponse des cellules du CaPAI envers d'autres modalités de traitement.

Les objectifs de ce projet de doctorat étaient d'innover dans le traitement du CaP et d'approfondir nos connaissances vis-à-vis les facteurs impliqués dans la progression clinique du CaP et la réponse thérapeutique. Comme tremplin pour atteindre ces objectifs, nous avons utilisé l'inositol hexakisphosphate (IP6), un agent phytochimique oralement non-toxique. Dans le chapitre 2, nous avons découvert que bien que l'IP6 présentait un activité anticancérese contre plusieu lignées du CaP, son effet était maximal contre les cellules du CaPAI n'exprimant pas le AR. Dans le contexte de la lignée cellulaire du CaPAI PC3 (AR-négative), l'efficacité de l'IP6 pouvait être modulée par l'expression stable du AR, indépendamment de la présence d'androgènes. En explorant ce phénomène, nous avons découvert que l'expression du AR empêchait l'apparition des manifestations de l'apoptose induites par l'IP6. De plus, l'expression du AR diminuait l'effet de l'IP6 sur l'expression d'un sous-groupe de gènes, impliquant potentiellement l'action de NF-κB et de protéines pro-apoptotiques de la famille BCL-2 tels NOXA et PUMA.

Suites à ces découvertes initiales, dans les capitres 3 et 4, nous avons utilisé des micro-étalages de tissus en combinaison avec l'immunohistochime, des méthodes d'analyse innovatrices, en plus d'algorithmes de modélisation multivariés émergeants (arbres de survie à base de partition récursive), pour évaluer l'omnipotence de NOXA, PUMA et du AR dans les tissus prostatiques. Pour la première fois, ces études ont identifié NOXA et PUMA en tant que cibles potentielles pour le traitement du CaP et ont établi NOXA comme étant un indicateur pronostique de RCB lorsque qu'évalué en combinaison avec certaines caractéristiques clinico-pathologiques. Ces études ont également souligné l'implication potentielle du AR localisé au noyau dans les étapes initiales de la progression du CaP. De plus, les résultats obtenus supportent l'étude future des fonctions cytoplasmiques et androgéno-indépendantes du AR, en vue de la prévalence du AR cytoplasmique dans le CaPAI et en vue de son utilité pronostique potentielle dans certains contextes.

Dans le chapitre 5, nous avons poursuivi l'exploration des mécanismes de l'IP6 dans le contexte des cellules du CaPAI PC3. Nous avons établi que, du moins dans cette lignée cellulaire, il est peu probable que l'action de l'IP6 implique NF-κB. Par contre, nous avons présenté des évidences supportant le rôle des protéines de la famille BCL-2 dans le mécanisme d'action de l'IP6. D'autant plus important, les informations générées au sujet des mécanismes de l'IP6 nous ont permis de rationaliser une stratégie pour augmenter l'efficacité de l'IP6 à l'aide d'inhibiteurs du protéasome, une option thérapeutique émergente pour le traitement du CaPAI. En effet, nous avons découvert qu'un traitement combiné à l'IP6 et aux inhibiteurs du protéasome augmente significativement la cytotoxicité observée, qui impliquerait la dépolarisation mitochondriale et potentiellement les membres pro-apoptotiques de la famille BCL-2.

Dans leur ensemble, les résultats présentés dans cette thèse de doctorat ont contribué au domaine du traitement du cancer de la prostate par l'entremise de découvertes de nouveaux marqueurs pronostiques et d'options thérapeutiques. Pris avec les recherches futures, nous croyons que les innovations avancés durant

de projet de doctorat pourraient éventuellement aider à mieux gérer le CaP et le CaPAI

Mots clés :

Cancer de la prostate
Inositol hexakisphosphate
Récepteur aux androgènes
Protéines de la famille BCL-2
Marqueurs pronostiques
Arbres de survie
Inhibiteurs du protéasome

SUMMARY

Prostate cancer (PCa) is the most frequently diagnosed cancer in North American men. When localized to the prostate, surgery and radiotherapy are often used successfully although a significant proportion of patients experience disease relapse (also termed biochemical recurrence or BCR). The mainstay for treatment of advanced or recurrent PCa is androgen-deprivation therapy (ADT), which aims to reduce the transcriptional activity of the androgen receptor (AR). Although ADT can prolong the life of PCa patients, androgen-independent (AI) PCa eventually arises, for which treatment is essentially palliative. Several studies indicate that there are both AR-dependent and AR-independent mechanisms involved in the resistance to ADT, which may also influence the efficacy of other treatment modalities. As such, there is a pressing need to improve our ability to match patients with the appropriate treatment and to increase the number and potency of clinically available therapeutic options for PCa and AIPCa patients.

The objectives of this doctoral thesis were to innovate in the treatment of PCa and to further our understanding of the factors involved in both clinical outcome and therapeutic response. Throughout the doctoral thesis, we have used inositol hexakisphosphate (IP6), an orally non-toxic phytochemical, as a stepping stone towards achieving these objectives. In chapter 2, we discovered that although IP6 presented anti-cancer activity against several PCa cell lines, its effect was greatest in AR-negative AIPCa cells. In the context of AR-negative PC3 AIPCa cells, the efficacy of IP6 could be modulated by stable expression of the AR, independently of the presence of androgens. In exploring this phenomenon, we discovered that the expression of the AR prevented the manifestations of apoptosis induced by IP6 as well as the up-regulation of a subset of genes, which indicated the potential implication of NF-κB and pro-apoptotic members of the BCL-2 family such as PUMA and NOXA.

Following these initial discoveries, in chapters 3 and 4 we employed tissue microarray technology combined with immunohistochemistry, innovative analysis methods, as well as emerging multivariate modeling algorithms (recursive

partitioning-based survival trees) to assess the omnipotence of NOXA, PUMA and the AR in prostate tissues. For the first time, these studies identified NOXA and PUMA as potential targets for PCa treatment and established NOXA as a promising prognostic indicator of BCR when included within multivariate survival tree models including key clinico-pathological features. These studies also highlighted the potential involvement of nuclear AR in the initial stages of PCa. In addition, given the observed prevalence of cytoplasmic AR in AIPCa patients and the finding that cytoplasmic AR may hold prognostic information in certain contexts; these studies provided further rationale for studying cytoplasmic androgen-independent functions of the AR.

In chapter 5, we continued to explore the mechanisms of IP6 in the context of PC3 AIPCA cells. We established that, at least in these cells, the action of IP6 does not likely involve NF-κB. However, we provided further evidence supporting a role of BCL-2 family members in mediating the effects of IP6. Most importantly, the information collected allowed us to rationalize a strategy to enhance the activity of IP6 using proteasome inhibitors, an emerging therapeutic option for AIPCa. Indeed, we discovered that combined treatment of AIPCa cells with proteasome inhibitors and IP6 leads to a significantly enhanced cytotoxicity involving mitochondrial depolarization and likely implicating pro-apoptotic BCL-2 family members.

Overall, the results presented in this doctoral thesis have contributed to the field of prostate cancer therapy through the discovery of both novel prognostic markers and therapeutic options. Altogether with future research, we believe that the innovations brought forth during the course of this doctoral project could eventually lead to the better management of PCa and AIPCa.

Key words:

Prostate cancer

Inositol hexakisphosphate

Androgen receptor

BCL-2 family proteins

Prognostic markers

Survival trees

Proteasome inhibitors

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER III

CHAPTER IV

LIST OF FIGURES

CHAPTER IV

CHAPTER V

LIST OF ABBREVIATIONS

17-AAG	17-Allylamino-17-Demethoxygeldanamycin
19S	sedimentation coefficient of 19
26S	sedimentation coefficient of 26
40S	sedimentation coefficient of 40
4E-BP1	eukaryotic elongation initiation factor 4E binding protein 1
60S	sedimentation coefficient of 60
A1	GM-CSF-inducible gene, A1
ABD	androgen-binding domain
ActD	Actinomycin D
ADT	androgen deprivation therapy
AF-1	activating function 1
AF-2	activating function 2
AI	androgen-independent
AIF	apoptosis inducing factor
AIPCa	androgen-independent prostate cancer
Akt	thymoma viral proto-oncogene / protein kinase B
ALLN	N-acetyl-leucinyl-leucinyl-norleucinal
ALT	alternative lengthening of telomeres
Aly	mutated in alymphoplasia
ANT	adenine nucleotide translocator
AP-2	Adaptor protein 2
AP-3/AP180	Adaptor protein 3 / 180
Apaf-1	apoptotic protease activating factor-1
APC	adenomatous polyposis coli
APC/C	Anaphase promoting complex or cyclosome
Ape-1	apurinic endonuclease-1
AR	Adrogen receptor
ARA	Andorgen receptor associated protein
ARE	androgen response element
ARF-BP1	ADP-ribosylation factor binding protein 1
ATF2	activating transcription factor-2
ATM	Ataxia telangiectasia mutated
ATP	Adenosine tri-phosphate
ATPase	Adenosine tri-phosphate phosphatase
ATR	Ataxia telangiectasia related
BAD	BCL-XL/BCL-2-associated death promoter homolog
BAK	BCL-2 antagonist/killer
BAX	BCL-2 associated protein X
BBC3	BCL-2 binding component 3
BCL-2	B-cell lymphoma protein 2
BCL-2L-10	BCL-2 like gene 10

BCL-B	B-cell lymphoma protein B
BCL-GL	B-cell lymphoma protein G long
BCL-GS	B-cell lymphoma protein G short
BCL-RAMBO	BCL-2 homolog rambo
BCL-W	B-cell lymphoma protein W
BCL-XES	B-cell lymphoma protein X extra short
BCL-XL	B-cell lymphoma protein X long
BCL-XS	B-cell lymphoma protein X short
BCR	biochemical recurrence
bFGF	basic fibroblast growth factor
BFL-1	BCL-2 homolog isolated from fetal liver 1
BH1	BCL-homology domain 1
BH2	BCL-homology domain 2
BH3	BCL-homology domain 3
BH4	BCL-homology domain 4
BID	BH3 interacting domain death agonist
BIK	BCL-2 homolog induced killer
BIM	BCL-2 interacting mediator of cell death
BMF	BCL-2-modifying factor
BMP7	bone morphogenic protein 7
BNIP1	BCL-2 homolog previously Nip-1
BNIP3	BCL-2 homolog previously Nip-3
BOD	BCL-2-related ovarian death gene
BOK	BCL-2-related ovarian killer
Boo	BCL-2 homologue of ovary
BPH	benign prostate hyperplasia
BRAG-1	brain-related apoptosis gene 1
BRCA1	breast cancer 1
CAD	caspase activated DNAse
caspase	cysteine aspartate protease
CBP	CREB-binding protein
cdc37	cell-division-cycle 37
CDK	cyclin dependent kinase
CDK1	cyclin dependent kinase 1
CDK2	cyclin dependent kinase 2
CDK4	cyclin dependent kinase 4
CDK6	cyclin dependent kinase 6
CDK7	cyclin dependent kinase 7
cDNA	complementary DNA
CHEK2	chekpoint kinase 2
CHOP-C	C/EBP homologous protein
CHX	cycloheximide
cIAP1	cellular inhibitor of apoptosis 1
cIAP2	cellular inhibitor of apoptosis 2

Cip	cdk inhibtor protein
Cip1	cdk inhibtor protein 1
Cip2	cdk inhibtor protein 2
CKII	casein kinase II
COXVb	Cytochrome oxidoreductase Vb
CREB	cAMP response element binding protein
c-Rel	cellular homolog of reticuloendotheliosis virus protein
DAG	diacylglycerol
DBD	DNA-binding domain
DBP5	DEAD-box protein 5
DD	death domain
DHT	dihydroxytestosterone
Diablo	direct inhibitor of apoptosis binding protein with low pI
dicer	bidentate RNaseIII family double-strand RNA endonuclease
DISC	Death-induced signaling complex
Diva	death inducer binding to vBCL-2 and Apaf-1
DNA	Deoxyribonucleic acid
DNA-PK	Deoxyribonucleic acid-dependent protein kinase
DNAse	Deoxyribonucleic acid nuclease
DR3	death receptor 3
DR-4	death receptor 4
DRE	digital rectal exam
$\Delta\Psi_m$	mitochondrial electrochemical gradient
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E2F	adenovirus E2 promoter binding factor
E2F1	adenovirus E2 promoter binding factor 1
E2F4	adenovirus E2 promoter binding factor 4
E3	ubiquitin ligase enzyme
EBP-α	Enhancer binding protein alpha
eEF	eukaryotic elongation factor
eEF2	eukaryotic elongation factor 2
EGF	epidermal growth factor
eIF	eukaryotic elongation initiation factor
eIF4E	eukaryotic elongation initiation factor 4E
eIF4G	eukaryotic elongation initiation factor 4G
ELAC2	elaC homolog 2
EMEA	European Agency for the Evaluation of Medicinal Products
EnR	endoplasmic reticulum
ER	estrogen receptor
ErbB	erythroblastosis erbB-related protein
ErbB-3	erythroblastosis erbB-related protein 3

ERK	extracellular response kinase
Ets	E26 transformation-specific
FADD	Fas associated protein with a death domain
Fas	Fas antigen
Fbw7	F-box protein with WD40 domains 7
FDA	Food and Dug Administration
FGF	fibroblast growth factor
FGFRiiib	FGF-receptor iiib
FHRL1	forkhead transcription factor like 1
G1	Gap 1
G2	Gap 2
Gle1	glucine-leucine-phenylanine-leucine lethal mutant
GSK3	Glycogen synthase kinase 3
HARAKIRI/DP5	Harakiri or death protein 5
HAT	Histone acetyl-tranferase
HDAC	histone de-acetylase
Hect	homologous to the E6-AP carboxyl terminus
HER-2/Neu/ErbB2	EGFR homolog 2/Neuregulin/erythroblastosis erbB-related protein 2
HIF-1α	Hipoxia induced factor 1 alpha
Hoxb13	Homeobox protein b13
HR	hormone refractory
HRPCa	hormone-refractory prostate cancer
Hsp90	Heatshock protein 90
IAP	inhibitor of apoptosis
IBS	integrated brier score
I^{CAD}	Inhibitor of caspase activated DNAse
IGF	insulin growth factor
IGFBP3	IGF-binding protein 3
IκB	Inhibitor of kappaB
IKK	inhibitor of kappa B kinase
IL-3	interleukin 3
IL-6	interleukin 6
INK4	inhibitor of cyclin-dependent kinase 4
INK4a	inhibitor of cyclin-dependent kinase 4 family member a
INK4b	inhibitor of cyclin-dependent kinase 4 family member b
INK4c	inhibitor of cyclin-dependent kinase 4 family member c
INK4d	inhibitor of cyclin-dependent kinase 4 family member d
Ins5P2K	1,3,4,5,6-pentakisphosphate 2-kinase
IP3	inositol-1,4,5 tri-phosphate
IP3R	inositol-1,4,5 tri-phosphate receptor
IP6	inositol hexakisphosphate
IRF-2	interferon regulatory factor 2
ITM2B (S)	Integral membrane protein 2B short

I-TRAQ	isobaric tags for relative and absolute quantification
JAK	Janus kinase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanide iodide
JNK	Jun Kinase
Ki67	Ki-67 antigen
Kip2	kinase inhibitor protein 2
Ku	Ku antigen
Ku70/80	Ku antigens 70/80
LH	lutheinizing hormone
LHRH	lutheinizing hormone releasing hormone
LPS	lipopolysaccharide
M	Mitosis
MAP-1	Modulator of Apoptosis 1
MAPK	mitogen activated protein kinase
MCL-1	Myeloid cell leukemia 1
MCL1-S	Myeloid cell leukemia 1short
mdm2	mini double-minute 2
MEK	Mitogen effector kinase
MG-132	Z-Leu-Leu-Leu-CHO
ML-IAP	Melanoma linked ihibitor of apoptosis
MMP	matrix metaloproteinase
MOMP	mitochondrial outer-membrane permeation
mRNA	messenger ribonucleic acid
MSH	MutS homolog
MTD	matador, a Spanish word for killer
mTOR	mammalian target of rapamycin
MudPIT	multidimensionl protein identification technology
Mule	MCL-1 ubiquitin ligase E3
NAD	nicotinamide adenine dinucleotide
NADH	Hydrogenated nicotinamide dinucleotide
NBK	natural born killer
NBS1	Nijmegen breakage syndrome 1
NCIC	National Cancer Institute of Canada
NF-κB	Nuclear factor tha binds kappa light chain immunoglobulin enhancer in B cells
NFX1	Nuclear factor that binds to X1 region of X box
NK	natural killer
NKX3.1	related to mouse NK-3 homeobox protein
NOXA	stands for damage
Nup	Nucleoporin
OCT1/2	octamer specific transcription factor 1/2
Omi/HtrA2	mammalian homolog of bacterial heatshock serine protease HtrA

P/CAF	p300/CBP-associated factor
p100	100 kilo Dalton protein
p107	107 kilo Dalton protein
p130	130 kilo Dalton protein
p15	15 kilo Dalton protein
p16	16 kilo Dalton protein
p18	18 kilo Dalton protein
p19	19 kilo Dalton protein
p21	19 kilo Dalton protein
p27	27 kilo Dalton protein
p50	50 kilo Dalton protein
p52	52 kilo Dalton protein
p53	53 kilo Dalton protein
p57	57 kilo Dalton protein
p65	65 kilo Dalton protein
p73	73 kilo Dalton protein
PABP	Poly adenosine binding protein 1
PARP	Poly(Adenine Dinucleotide Phosphate-ribose) polymerase
PCa	prostate cancer
PCNA	proliferating cell antigen
PCR	polymerase chain reaction
PDEF	prostate-derived Ets transcription factor
PDGF	platelet-derived growth factor
PDGFR	platelet derived growth factor receptor
PDK	phosphatidylinositol-dependent kinase
PDK1	phosphatidylinositol-dependent kinase 1
PEST	proline-apartate-serine-threonine
PI3K	Phosphatidyl-inositol-3-kinase
PIDD	p53-induced protein with a death domain
PIN	prostatic intra-epithelial neoplasia
PIP2	phosphatidyl-inostol (4,5) bi-phosphate
PIP3	phosphatidyl-inosoitol (3,4,5) tri-phosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PolyA	poly adenosine
PP1	protein phosphatase 1
PP2A	protein serine/threonine phosphatase 2A
PP3	protein phosphatase 3
pRb	retinoblastoma protein
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
Ptc	Patched

PTEN	phosphatase and tensin homolog
PUMA	p53-up-regulated mediator of apoptosis
pVHL	Voh Hippel-Lindau protein
RAIDD	RIP-associated ICH-1/CED-3-hmologous protein with a death domain
RAN	Ras-related nuclear GTPase
REF	Ribonucleic and export factor
RelA	reticuloendotheliosis virus protein homolog A
RelB	reticuloendotheliosis virus protein homolog B
RING-H2	Zinc finger family protein motif
RISC	Ribonucleic acid induced silencing complex
RNA	Ribonucleic acid
RNAse	Ribonucleic acid nuclease
RNAse L	Ribonucleaic acid nuclease L
ROC	Receiver operating characteristic
ROCK-1	Rho kinase 1
RP	radical prostatectomy
RPART	recursive partitioning regression trees
rRNA	ribosomal ribonucleic acid
RTK	receptor tyrosine kinase
S	Synthesis
SCF	Skp-Cul-F-box complex
$SCF\beta^{TRCP}$	Skp-Cul-F-box complex containing Beta-tranducin repeat containing protein
SELECT	selenium and vitamin E chemoprevention trial
shRNA	short hairpin ribonucleic acid
siRNA	short interfering ribonucleic acid
Skp1	Shaggy kinase in Schizosaccharomyces pombe 1
Skp2	Shaggy kinase in Schizosaccharomyces pombe 2
Smac	Second mitochondrial activator of caspase
SMAD	related to Sma and mothers against dpp
SPIKE	small protein with inherent killing effect
SRC-1	steroid receptor coactivator-1
STAT	signal transducer and activator of transcription
Succ-LLVY-AMC	Succinate-Leu-Leu-Val-Trp-amino-4-methylcoumarin
SUV39H1	Su(var) or supressor of variability 3-9 homolog 1
SUV39H2	Su(var) or supressor of variability 3-9 homolog 2
TAP	Tip of herpesvirus saimiri-associated protein
tBID	truncated BID
TFIIH	Transcription factor II H
TGF-α	transforming growth factor-alpha

TGF-β	Transforming growth factor beta
TMRPSS2	transmembrane protease serine 2
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor receptor 1
TNM	tumor, lymph nodes, metastasis
TOM	translocase of the outer membrane
TPA	tetradecanoylphorbol 13-acetate
TRADD	TNF-receptor associated protein with a death domain
TRAIL	TNF-receptor associated apoptosis inducing ligand
Trail-R1	Trail receptor 1
TRAMP	transgenic adenocarcinoma of the mouse protate
tRNA	transfer ribonucleic acid
TURP	trans-urethral resection of the prostate
Uba1	Ubiquitin activating enzyme 1
UBC	Ubiquitin conjugating enzyme
UVB	Ultraviolet radiation B
VDAC	voltage-dependent anion channel
VEGF	vascular endothelial growth factor
VSV	vesicular stomatitis virus
Waf1	Wid-type p53-activated fragment 1
Wnt	Drosophila melanogaster segment polarity gene wingless/ int-1
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disfonate
xIAP	x-linked inhibitor of apoptosis
Z-ARR-AMC	z-Ala-Arg-Arg-amino-4-methylcoumarin
Z-LLE-AMC	z-Leu-Leu-Leu-Asp-amino-4-methylcoumarin
ZVAD-fmk	z-valine-alanine-asparagine-fluoro-methyl-ketone

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CHAPTER I

1. Introduction

Over the last century, cancer has emerged as one of the most problematic diseases of the western world. With the advent of globalization and the increasing life expectancy worldwide [1], cancer is likely to become one of the most significant challenges to human medicine for centuries to come. In this chapter, we will begin by briefly reviewing the maintenance of cellular homeostasis and explore how this homeostasis is fundamentally altered in cancer cells. We will subsequently focus on the development of prostate cancer and its current treatment. Emerging therapeutic concepts in prostate cancer will then be introduced and the potential of inositol hexakisphosphate will be showcased within this context.

1.1 Cellular Homeostasis

Cellular homeostasis can be defined as the equilibrium between production and destruction of cellular components. Although the cell processes a wide range of molecules, we will focus on the homeostasis deoxyribonucleic acids (DNA), of ribonucleic acids (RNA), and of proteins, as they are intimately linked and as they are of central importance for the development of cancer.

1.1.1 DNA Homeostasis

Chromosomal DNA is considered to be the primary holder of the genetic information within the cell and acts as a template for the production of RNA and proteins. The specific sequence of DNA within chromosomes constitutes the template for RNA synthesis and subsequent protein synthesis. This intimate relationship is of utmost importance for the cell, as changes in the DNA sequence may have a severe impact on cellular functions and consequently on the overall cellular homeostasis.

1.1.1.1 DNA Replication and DNA Degradation

In the normal cell, production of DNA through DNA replication is a tightly regulated process, allowed to occur only during the synthesis or S phase of the cell cycle (see section 1.2.1) [2]. This process is mediated by the action of various proteins including DNA primases, DNA ligases, DNA helicases,

topoisomerase, and finally DNA polymerases that play a most direct role by synthesizing a complementary DNA strand from a single strand template [3]. Counter to DNA polymerase activity, DNAses act to break down the DNA polymer into smaller sub-components. Exonucleases effectively digest DNA from one end to the other (either 5' to 3' or 3' to 5') while endonucleases cut within a DNA strand. The action of endo and exo-nucleases is important for diverse processes such as DNA repair (eg. Ape-1 [4], see below) and apoptosis (eg. CAD [5], see section 1.2.3.3.2).

1.1.1.2 Genome Integrity and the Impact of DNA Mutation

It is of utmost importance for the cell to maintain the integrity of the DNA sequence as any unwarranted change can potentially lead to the disruption of cellular homeostasis. In addition, this disruption may be transmitted to daughter cells following cell division. As such, various proteins (that are synthesized based on the DNA sequence, see section 1.1.3.1) are normally responsible for maintaining chromosomal integrity. These include enzymes involved in the repair of DNA single-strand breaks [eg. Ape-1, [4], PARP [6]], or double-strand breaks [e.g ATM, [7], DNA-PK and Ku proteins [8]], DNA mismatches [e.g, MSH, PCNA [7]], or that maintain the stability of DNA ends or telomeres (e.g Telomerase [9]). Other proteins such as p53 act as genome guardians and coordinate cellular response to DNA damage [10]. The proper regulation of these proteins is therefore also critical for maintaining a stable genome (see section 1.2.4.2.2).

1.1.2. RNA Homeostasis

RNA is to DNA as paper is to a paper press and therefore any change that is made to the DNA template is automatically transferred unto the corresponding RNA message. The process of making RNA from DNA is referred to as transcription and occurs mainly in the nucleus and within the Gap 1 (G1), S, and Gap 2 (G2) phases of the cell cycle [11] (see section 1.2.1.). As RNA is subsequently translated into proteins that play major structural and functional roles within the cell (see section 1.1.3.1), the tight regulation of transcription is extremely important for maintaining cellular homeostasis. As such, many cell-

signaling pathways are devoted to the transcriptional control of genes (see sections 1.2.2.1, 1.2.3.2.5, 1.2.4.1, and 1.3.5.5).

1.1.2.1. RNA Transcription and its Regulation

Transcription is regulated both by DNA and protein. Specific sequences in DNA (e.g gene promoters and enhancers) act as binding sites for transcription factors that initiate or activate the process of RNA synthesis through the action of RNA polymerases [e.g RNA Polymerase I [12], II [13], III [14]]. As such, DNA mutations that modify either the specificity of the DNA sequences recruiting transcription factors or that modify the specificity of transcription factors themselves can have a major impact on RNA homeostasis.

The condensation state of chromatin is one of the most fundamental regulators of gene expression. In its most basic form, chromatin is composed of negatively charged DNA wrapped around positively charged histone proteins (altogether called a nucleosome). Condensed chromatin (heterochromatin) is much less accessible to transcription factors, RNA polymerases, and their associated co-factors. Consequently, gene transcription generally occurs within loose chromatin domains (euchromatin) [15, 16].

Whereas the DNA sequence regulates which transcription factors (including RNA polymerases) bind where, histone proteins are the structural regulators of the chromatin condensation state. Histone proteins can be post-translationally modified by covalent addition of various moieties including acetyl, methyl, phosphoryl, and even ubiquitin groups. Post-translational modification of histones can lead to the alteration of chromatin structure. These modifications occur on specific histone residues and altogether constitute a histone code that acts in parallel to the genetic code provided by the DNA sequence [16].

Although researchers are finding the histone code to be increasingly complex [16, 17], it is generally considered that the acetylated state of histones is associated to transcription permissive euchromatin whereas de-acetylated histones are rather associated to repressive heterochromatin. Importantly, histone acetyl tranferases (HATs) mediate the addition of acetyl groups whereas histone de-

acetylases (HDACs) mediate the removal of these moieties. In effect, activator transcription factors often act to recruit HATs while repressor transcription factors recruit HDACs [18].

1.1.2.2. RNA Processing and Degradation

Once RNA is produced, it is subsequently processed and spliced into its mature form (mRNA), void of intron sequences and equipped with a 5' methylguanosine cap and a 3' poly-adenosine (PolyA) tail. While the 5' methylguanosine cap plays a role in the initiation of protein translation (see section 1.1.3.1) both the 5' methyl cap and the PolyA tail play a role in protecting the RNA from degradation by RNAses, which oppose the action of RNA polymerases and promote RNA de-polymerization [11]. Recently, it has been found that the cell possesses a highly specific system of RNAses that permits the degradation of mRNA in sequence-specific manner. The RNA-induced silencing complex (RISC) in concert with the RNA endonuclease dicer induces the degradation of specific mRNAs. Notably, exploitation of this endogenous cellular machinery has spawned the emergence small interfering RNA (siRNAs) and short hairpin RNA (shRNAs) technology that allows for the specific down-regulation of genes and their associated proteins using short complementary RNA sequences [19, 20] (see chapter II).

1.1.3. Protein Homeostasis.

The main purpose of mRNAs is to act as an intermediate template for the production of protein through a process termed translation. This process is mediated by the ribosome, a large ribonucleoprotein complex composed of two smaller subunits (40S and 60S). Much like RNA production, protein translation occurs mainly in the G1, S, and G2 phases of the cell cycle [11]; however, unlike RNA transcription, this process occurs mainly in the cytoplasm and endoplasmic reticulum, although more restricted translation also occurs in other organelles such as the mitochondria [21] (see section 1.2.3.1) and the nucleolus [14, 22]. Notably, the nucleolus is the primary site for ribosomal RNA transcription and ribosome maturation [22].

One of the first control points for protein production is mRNA export from the nucleus, which proceeds through the TAP/NFX1 pathway and involves a large number of proteins including nuclear export receptors (eg. TAP/NFX1), nuclear export adaptors (Aly/REF), ATPases/RNA helicases (eg. DBP5) and nucleoporins (eg. Gle1 and Nup family proteins) [23]. The second major control point for protein production from mRNA involves translation initiation and recruitment of the 40S ribosome. This involves numerous elongation initiation factors (eIF) such as eIF4E that binds the 5' methylguanosine cap, eIF4E binding proteins (e.g 4E-BP1), and eIF4G that also causes circularization of mRNA indirectly by its interaction with poly(A) binding protein (PABP1). Although regulation at the translation initiation step is better described, protein translation can also be regulated at the elongation and termination steps through the action of elongation factors (eEFs) such as eEF2 [24]. As protein production can have rather immediate functional consequences for cellular homeostasis, protein translation is also tightly regulated through the concerted action of many signaling pathways, one of which is the mTOR pathway ([25], see section 1.2.2.2),

1.1.3.2. Protein Degradation and the Ubiquitin/Proteasome System

Although lysosomal proteases account for 10-20% of cellular protein degradation [26], the 26S proteasome is the principle mediator of protein degradation responsible for the ATP-dependent processing of 80-90% of cellular proteins [27]. The 26S proteasome is a large 2000 kDa multiprotein complex composed of a barrel-shaped 20S subunit and two "lid" 19S subunits. The 20S subunit contains several proteolytic sites within its two central β-rings and possesses trypsin-like, chymotrypsin-like and caspase-like protease activity. Notably, the chymotryptic-site is thought to be rate limiting in proteasome-mediated degradation [27]. The 19S subunits are mainly regulatory in nature and direct proteasomal degradation by binding poly-ubiquitinylated proteins that, as such, have been targeted for degradation. Thus, the targeting of proteins for proteasome-mediated degradation by the process of poly-ubiquitination is a key regulatory step of protein homeostasis and can profoundly impact cellular homeostasis.

The ubiquitination of cellular proteins is mediated by a series of three types of enzymes, namely E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating proteins, and E3 ubiquitin ligases [28, 29]. While mono-ubiquitination can play a role in altering protein localization [30] and is increasingly thought to play a role in chromatin organization [31], poly-ubiquitinylation (4 ubiquitin subunits or more) targets proteins for proteasomal degradation [28]. While only one E1 enzyme (Uba1) is required to conjugate ubiquitin to a small number of cellular E2 enzymes (UBCs) that have limited specificity, there are several E3 ubiquitin ligases (eg. Skp1/2, APC/C, mdm2, $SCF\beta^{TRCP}$) that confer most of the substrate specificity as well as temporal and spatial control to the ubiquitin/proteasome system [28, 29, 32]. The E3 ubiquitin ligases contain either Hect or RING-H2 domains and are notably involved in a variety of cellular processes including genomic surveillance (e.g mdm2 [33] see section 1.2.2.2 and 1.2.4.2), cell signaling (e.g $SCF\beta^{TRCP}$[34], see section 1.2.4.1.3), cell cycle (eg. SCF (skp1/2) [35, 36], APC/C [37], see section 1.2.2.3) and cell death (e.g. Mule/ARF-BP1[38] see section 1.2.3.2.6).

1.2.Disruption of Cellular Homeostasis: Cell cycle, Apoptosis, and Cancer

By definition, the homeostatic state is a stagnant balance between import, export, production, and degradation of each component of the cell. In reality, this balance is in continual reassessment. As the cell reacts to various stresses and stimuli, the homeostatic balance is perturbed and the state of the cell is altered. As we will soon discuss, the outcome of these perturbations depends on both the stimulus and the state of the cell.

1.2.1.The Cell Cycle

The cell cycle can be viewed as a programmed and controlled alteration of cellular homeostasis that leads to cytokinesis and cellular proliferation. The cell cycle is divisible in four stages, G1, S, G2, and M (mitosis) [11]. These stages are associated to distinct cellular activities (see sections 1.1.1-1.1.3) and are periodically regulated at "checkpoints" that play a crucial role in overseeing the replication of the genome and its proper distribution within the resulting daughter

cells. The cell cycle checkpoints also play a crucial role in the control of cellular proliferation within the organism, as they can be regulated by growth factors and various extracellular signals [39](Refer to Chapter I Figure. 1 for an overview).

1.2.1.1 Cyclin-Dependent Kinases

The cell cycle is controlled by a complex network of proteins of which the main constituents are cyclins and cyclin-dependent kinases (CDK). Although there are at least nine known CDKs, five of them play an active role throughout the cell cycle. Specifically, CDK 4, 6, and 2 are active during the G1 phase, CDK2 is active in S phase while CDK1 is active in both G2 and M phases. CDK7 is active at all cell cycle phases. Although the kinase activity of CDK6, 4, 2 and 1 changes throughout the cell cycle, their protein levels generally remain stable. CDK activity is regulated by cyclins, whose levels rise and fall periodically throughout the cell cycle [40-42].

1.2.1.2. Cyclins

Much like CDKs there are over sixteen identified cyclins although many of them are not related to the cell cycle. cyclins D1, D2 and D3 are expressed maximally during the G1 phase and associate to CDK4 and CDK6. cyclin E is expressed during the G1/S phase transition and associates with CDK2. The activity of CDK2 following the G1/S transition is maintained by its association with cyclin A, which is expressed throughout the S phase, progressively decreasing until the G2/M phase transition where it interacts instead with CDK1. During the initial stages of M phase, cyclin B levels are at their highest, leading to sustained activation of CDK1 until the cycle repeats itself [41]. Notably, in contrast with cyclins E, A and B, D-type cyclin expression is regulated by growth factor signaling [42]. Importantly, CDK/cyclin complexes have varying specificities and can phosphorylate a wide range of proteins that promote changes in cellular organization, cell cycle progression, and eventually cell division.

1.2.1.3 Cell Cycle Checkpoints and Cell Cycle Inhibitors

Acting as natural "brakes" for the cell cycle and playing important roles at the cell cycle checkpoints, CDK inhibitors block the activity of CDK/cyclin complexes. The better-known CDK inhibitors fall into two major families. The INK4 family comprises p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) and present ankyrin repeat domains. INK4 family CDK inhibitors are generally thought to compete with D-type cyclins to block the cell cycle in G1. The Cip/Kip family comprises p21(Waf1/Cip1), p27(Cip2), p57(Kip2) and also share a common inhibitory domain. Although Cip/Kip family CDK inhibitors act on cyclin D/CDK complexes *in vitro*, they preferentially act on CDK2 complexes *in vivo* [41, 43].

Although INK4 and Cip/Kip family proteins are more or less direct CDK inhibitors, a third category of cell cycle inhibitors, the pocket proteins, mediate their effect through E2F transcription factors. E2F family transcription factors are key regulators of the G1/S cell cycle transition but are also involved in cell differentiation and apoptosis [44, 45] (see section 1.2.3.2.5). This family of proteins consists of at least seven members, of which E2F1, 2 and 3 are considered transcriptional activators that act upon release from their interaction with pRb: the prototypical pocket protein family member. While the function of the other E2F family members is more obscure, E2F4 and E2F5 are thought to act as transcriptional repressors when bound to pocket proteins (including pRb, p107 and p130) that also recruit HDACs. Among others, Rb proteins can be phosphorylated by CDK(4/6)/cyclin D or CDK2/cyclin E complexes during cell cycle progression. Phosphorylation of pRb family members results in their dissociation from E2F binding partners. The coordinated activity of E2F members results in the modulation of E2F-regulated gene expression and cell cycle progression [44, 46-48].

1.2.2. Regulation of the Cell Cycle.

Net cyclin and CDK inhibitor expression at the protein level depends on mRNA availability, protein production, and protein degradation. As such this offers several layers of regulation for cell cycle control.

1.2.2.1 Transcriptional Control of Cell Cycle Components

The first level of control occurs at the level of mRNA transcription. For example, cyclin D1 mRNA expression is regulated by various transcription factors including NF-κB [49] and β-catenin [50]. Upstream signaling pathways in turn regulate the activity of these transcription factors, such as that of PI3K/Akt/IKK (NF-κB) and Wnt/GSK3 (β-catenin) (see sections 1.2.4.1.1 to 1.2.4.1.4). Moving forward in the cell cycle, cyclin E can be up regulated by E2F1 following hyper-phosphorylation of pRb [51]. In contrast, to halt cell cycle progression, the expression of the p15 (INK4b) CDK inhibitor can be up regulated at the mRNA level following cell stimulation by TGF-β [52]. Similarly, the p53 transcription factor can induce p21 (Waf1/Cip1) mRNA up-regulation in response to DNA damage [53].

1.2.2.2. mTOR and the Translational Control of Cell Cycle Components

One of the better-described pathways that regulate cell cycle and other proteins at the translation level is the mTOR (mammalian target of rapamycin) pathway. As its name implies, mTOR was first identified as a target of rapamycin,

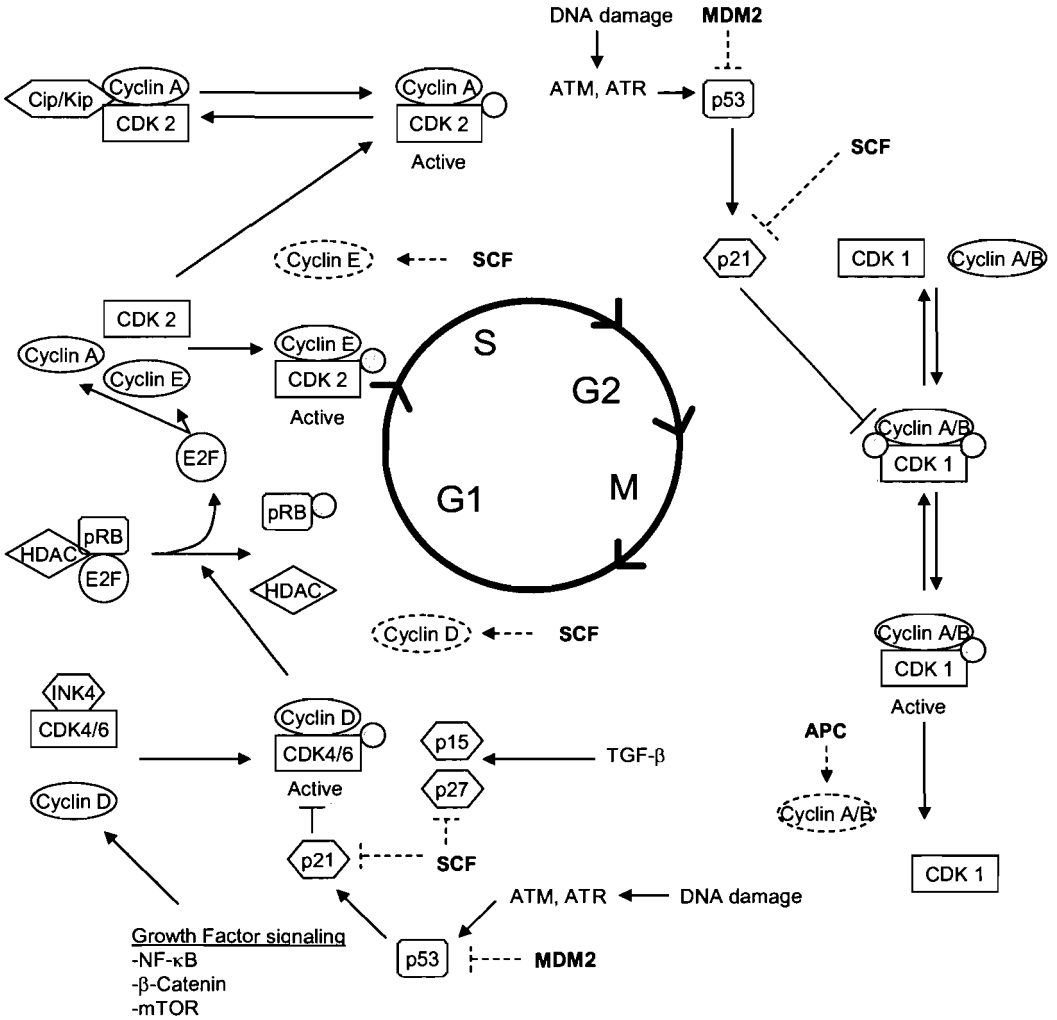

Figure 1. Overview of the cell cycle and its regulators. Refer to sections 1.2.2 through 1.2.2.3. Grey circles indicate a phosphorylation event whereas dashed lines indicate proteasome-mediated degradation. Lines ending with bars indicate an inhibitory effect. Ubiqutin ligases are highlighted in bold. Adapted from [41].

a drug that inhibits cell proliferation [54]. In normal cells, the mTOR pathway is typically initiated by an increased availability in amino acids or by upstream activation of PI3K and Akt. mTOR activation has been shown to lead to the inhibition of 4E-BP1 that normally represses translation by binding eIF4E. Hence, activation of mTOR promotes translation of various proteins including cyclin D1 whereas inhibition of mTOR leads to decreased protein production and cell cycle arrest [25].

1.2.2.3. Proteasome-Mediated Degradation of Cell Cycle Components

Another important cell cycle regulation mechanism is proteasome-mediated degradation of cyclins and other proteins involved in cell cycle control. As mentioned previously, this involves several E3 ubiquitin ligases. SCF (skp/ cul / F-box protein) E3 ligase complexes play an important role to this effect, mediating the degradation of CDK inhibitors such as $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ [32, 55] as well as of cyclin D1 [56] and cyclin E [57]. Notably, the composition of the SCF complex defines its target specificity. For example SCF1 (skp1/cul1/F-box protein) complexes alter specificity through the changing of the F-box protein. Skp2, Fbw7 and β-TrCP are the best known F-box proteins and while skp2 and fbw7 play direct role in the cell cycle, SCF complexes containing β-TrCP play a more indirect role by controlling cell signaling through the NF-κB and β-catenin pathway [32, 34, 50] (see sections 1.2.4.1.3 and 1.2.4.1.4). In a similar fashion to SCF complexes, the APC (Anaphase Promoting Complex) ubiquitin ligase mediates the degradation of cyclin A and B [58]. Other important E3 ubiquitin ligases include MDM2, which controls the expression of the genome surveillance factor p53 [59].

1.2.3. Apoptosis.

If the cell cycle can be viewed as a controlled perturbation of cellular homeostasis leading to cell division, then apoptosis may be described as a controlled response to the disruption of cellular homeostasis leading to cell death.

Likely aiming at containing cellular toxins that may be damageable towards surrounding cells, the apoptotic cell undergoes distinct morphological and biochemical changes. These typically include cell shrinkage, cell membrane blebbing and presentation of phosphatidyl-serine at the cell surface, loss of nuclear envelope integrity, chromatin condensation, and DNA fragmentation [60, 61]. The apoptotic program can be initiated by both intracellular (intrinsic) and extracellular (extrinsic) signaling pathway [62]. Apoptosis is a highly regulated process and its progression involves a cascade of molecular events, implicating a myriad of proteins including pro- and anti-apoptotic proteins (see sections 1.2..3.2.1 to 1.2.3.2.3), inhibitors of apoptosis (IAPs, see section 1.2.3.3.3), proteases (see section 1.2.2.3.1) and DNAses (see section 1.2.3.3.2) [63, 64] (see Chapter I Figure. 2 for an overall schematization). In the context of tissues and organs, the process of apoptosis is extremely important as it counterbalances the effects of cell division, thereby maintaining tissue homeostasis or actively sculpting organ shape [65, 66].

1.2.3.1 The Mitochondria

Before we begin to review the molecular details of apoptosis and the processes of cell death, it is important to describe one of its central regulatory organelles: the mitochondrion. The mitochondrion possesses its own DNA, leading some to suggest that it may have evolved separately from the cell and was later incorporated in a symbiotic relationship [67]. Importantly, mitochondria not only provide eukaryotic cells with energy by acting as the main sites of ATP (adenosine tri-phosphate) production, but also act as key regulators of apoptosis and cell death in general [64, 67].

1.2.3.1.1. Mitochondrial Energy Production

The mitochondrion is composed of an outer membrane and a larger ruffled inner membrane encircling the mitochondrial matrix. While the outer membrane acts as a gate between the cytosol and the mitochondria, the inner membrane plays a major role in the production of ATP, holding all the components of the

electron transfer chain. The electron transfer chain mega-complex is responsible for the establishment of a proton gradient between the inter-membrane space and the mitochondrial matrix. It is composed of several sub-complexes including the nicotinamide adenine dinucleotide (NAD) dehydrogenase complex, iron-sulfur proteins, Co-enzyme Q (ubiquinone), cytochrome B complex (B/C1), cytchrome C and also cytochrome oxydase (cytochrome A/A3 complex) [68]. Notably, whereas most of these complexes reside within the inner-mitochondrial membrane, cytochrome C sits in the inter-membrane space. The process of ATP production by ATP synthase, which sits on the matrix side of the mitochondrial inner membrane, is driven by the proton motility force that results from the pH gradient between the outer/inner membrane space and the mitochondrial matrix. [64, 67, 68]. As the inter-membrane space is more acidic than the mitochondrial matrix, this yields an electrochemical gradient referred to as $\Delta\Psi_m$[64].

1.2.3.1.2. Mitochondrial and Cellular Inter-Dependence

Within the mitochondrial matrix lays the mitochondrial genome, which is circular and much smaller than the nuclear genome (roughly 16 000 bp) and encodes for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 polypeptides. The mitochondrial matrix also harbors its own translation-competent ribosomes, although these are of significantly different composition than cytosolic ribosomes [21, 69] (see section 1.1.3.1). However, the mitochondrion is not auto-sufficient in that it requires the import of proteins encoded by nuclear genes, which are likely imported both post and co-translationally (e.g as they traverse the outer-membrane) [70]. Mitochondrial import of RNA has also been described, particularly for transfer RNAs [71], although the mechanisms involved are much more obscure than those involved in protein import, that are known to proceed through the action of the Translocase of the Outer Membrane (TOM) pore complex [72].

1.2.3.1.3. VDAC/ANT Complexes and the Inter-Membrane Space

Another key mitochondrial membrane pore complex involved in the transport of diverse molecules is VDAC/ANT. This complex is formed of VDAC

(Voltage-dependent anion channel) which lies within the outer membrane, ANT (adenine nucleotide translocator) located in the inner membrane, and cyclophilin D that associates to the matrix portion of ANT. In addition, benzodiazepine receptor and hexokinase, an enzyme involved in glucose phosphorylation, are associated to VDAC on the cytosolic side of the channel. [73-80]. Although this remains debated, some studies suggest that this highly regulated inner and outer membrane spanning channel plays a role in apoptosis because it allows for the dispersion of known pro-apoptotic factors that are present in the inter-membrane space. As will be discussed subsequently, these include cytochrome C that binds Apaf-1 and activates the apoptosome [81, 82], Apoptosis inducing factor (AIF) that plays several roles including chromatin condensation and DNA fragmentation [83], Diablo/Smac and Omi/HtrA2, both antagonizers of IAPs [84-86], EndoG that activates caspase-activated DNAse (CAD) [87, 88], as well as caspases 2 and 9 [89-92] (see section).

1.2.3.2. The Regulation of Mitochondrial Outer Membrane Permeability.

Given the presence of pro-apoptotic factors within the inter-membrane space and given the importance of the maintenance of the proton gradient and $\Delta\Psi_m$ for ATP production by ATP synthase in the inner membrane, outer mitochondrial membrane permeability is an extremely well regulated process. In fact, a whole family of proteins is dedicated to its control. The BCL-2-family of proteins is characterized by the presence of BCL homology domains (BH1, BH2, BH3 or BH4) and presently contains over 30 known members. These are generally categorized according to whether they promote or prevent apoptosis and how many (and which) BH domains the proteins contain [93].

1.2.3.2.1 Anti-Apoptotic BCL-2 Family Members

Anti-apoptotic proteins of the BCL-2 family ultimately prevent the permeation of the mitochondrial outer membrane and generally contain at least two different BH domains. These currently include the prototypical BCL-2 [94], BCL-XL [95], BCL-XES [96], BCL-W [97], MCL-1 [98], A1/BFL-1 [99], BRAG-1 [100], BOO/DIVA [101], BCL-B [102] and BCL-2L-10 [103].

1.2.3.2.2. Multi-Domain Pro-Apoptotic BCL-2 Family Members

In contrast to anti-apoptotic BCL-2 proteins, pro-apoptotic proteins of the BCL-2 family lead to the leakage of apoptosis effectors residing within the inter-membrane space into the cytoplasm (as described in section 1.2.3.1). These fall into two sub-categories based on whether they contain multiple different BH domains (multi-domain) or only one or sometimes two BH3 domains (termed BH3-only). Known multi-domain BCL-2-family members include BAX [104], BAK [105], BCL-XS [95], BOK/MTD [106], BCL-RAMBO [107] and BCL-GL [108]. Importantly, some of these proteins such as BAX, BAK and BID (see below) can form pore structures and permeate synthetic liposomes *in vitro* and may permit direct mitochondrial outer membrane permeation [109].

1.2.3.2.3. BH3-Only Pro-Apoptotic Proteins

Currently, known BH3-only proteins include BAD [110], BID [111], BIK/NBK [112], BIM/BOD [113], HARAKIRI/DP5 [114], BNIP1 [115], BNIP3 [116], PUMA/BCB3 [117], NOXA [118], BCL-GS [108], MCL1-S [119], BMF [120], MAP-1 [121], ITM2B(S) [122] and SPIKE [123]. It is generally thought that the major role of these proteins is to antagonize anti-apoptotic members of the BCL-2 family, although some such as BID and PUMA, may more directly promote pore formation mediated by multi-domain pro-apoptotic proteins such as BAX and BAK [124].

1.2.3.2.4. Control of outer membrane permeability by BCL-2-family proteins

This long list of pro and anti-apoptotic factors of the BCL-2-family gives us a general sense of the importance and complexity of mitochondrial membrane permeability control. Overall, it is thought that the balance of active pro- and anti-apoptotic proteins within the cell dictates the release of apoptogenic factors from the inter-membrane space of the mitochondria. Hence, we can invoke the notion of homeostasis to explain the action of BCL-2 family members on the mitochondria. For example, if the cell shifts its production towards accumulation of pro-apoptotic proteins, cells can survive in conditions where they would

normally die. In contrast, if cells accumulate pro-apoptotic proteins, they are rendered sensitive to various insults.

However, this model is somewhat of an over-simplification as it is clear that different BCL-2 family members play different roles in the cell, even though there appears to be considerable functional redundancy within BCL proteins [125]. Since most of these proteins were discovered in the past ten years (some like SPIKE were discovered less than five years ago) the details of their interactions are only beginning to be understood. It is clear from most studies that the multi-domain pro-apoptotic proteins, particularly BAX and BAK, play a central role. While BAK resides mainly in the mitochondrial outer membrane and homo-oligomerizes in response to a death stimulus [126], BAX is found mainly in the cytoplasm as a monomer. BAX can subsequently translocate to the outer mitochondrial membrane following a variety of pro-death stimuli where it may homo-oligomerize similarly to BAK, leading either directly or indirectly (e,g through VDAC/ANT) to mitochondrial permeation (see section 1.2.3.1.3) [127-129].

How BCL-2-family anti-apoptotic proteins prevent mitochondrial permeation and how BH3-only proteins promote it is currently a subject of intense debate. For example, one model suggests that anti-apoptotic proteins directly stabilize the mitochondrial membranes whereas BH3-only proteins actively destabilize it upon outer membrane attachment [130-134]. Other models propose that BCL-2-family proteins help to control what is termed the mitochondrial permeability pore, by regulating VDAC/ANT, thought to be one of its core components [135, 136]. In light of the interaction between the BH1-2-3 domains of anti-apoptotic proteins such as BCL-2 with the BH3 domain of multi-domain pro-apoptotic proteins, some have suggested that anti-apoptotic proteins directly antagonize multi-domain pro-apoptotic proteins. In this context, it is supposed that BH3-only proteins can release and further activate BAX/BAK such as has been suggested to be the case for BID [137, 138]. Notably, BID and PUMA have been shown to interact with the first alpha-helix of BAX, an interaction which was necessary to mediate BAX-induced mitochondrial depolarizarion [139].

On the other hand, evidence suggests that anti-apoptotic proteins such as BCL-2, BCL-XL and MCL-1 would associate to and prevent the action of BH3-only proteins such as BID and PUMA that directly activate BAX/BAK. Recent studies suggest that BH3-only proteins interact with specific anti-apoptotic proteins [124, 140, 141]. In one study using full length and mutant BH3-only proteins *in vitro* and *in vivo*, it was shown that the varying affinities of BH3-only proteins towards the anti-apoptotic family members leads to hierarchical interactions. For example, whereas NOXA disrupts the interaction of PUMA, BID and BIM with MCL-1, the binding of BAD disrupts the interaction of activator BH3-only proteins with BCL-2/BCL-XL [124].

1.2.3.2.5. Transcriptional Control of BCL-2 Family Members

At this point, we can begin to appreciate that the regulation of BCL-2-family member expression and their activation is a key component of the apoptotic process. A number of transcription factors are known to modulate the expression of BCL-2-family members, often in a cell type specific manner. For example, NF-κB binding sites are found in the BCL-2 promoter and over-expression or activation of NF-κB can increase BCL-2 expression in various contexts including leukemia and breast cancer cells [142, 143]. NF-κB is also thought to regulate the expression of BCL-XL, although STAT and Ets-family transcription factors may also play a role [144]. In myeloid cells, the JAK/STAT-3 pathway is thought to control MCL-1 expression in response to IL-6 [145].

With respect to pro-apoptotic proteins, NOXA and PUMA were initially discovered as targets of the p53 transcription factor [117, 118]. It was later observed that the p53 homolog p73 could regulate the expression of PUMA [146] and that transcription factors of the E2F family (particularly E2F1) could induce NOXA and PUMA expression [147]. In addition, the expression of NOXA and BIM can be up regulated by forkhead family factors such as FKHRL1 [147, 148]. Finally, it was recently found that CHOP-C/EBP-α mediates the up-regulation of BIM in response to endoplasmic reticulum (EnR) stress [149].

Alternative splicing can also yield some form of control in the production of pro and anti-apoptotic proteins. Skipping of MCL-1 exon II yields MCL-1S, which acts as a pro-apoptotic apoptotic protein in contrast with the full-length form [119]. Similarly BIM has several isoforms, one of the smaller ones being BIM-AD, which lacks the domain that normally allows the sequestering of BIM at the cytoskeleton, resulting in a highly potent pro-apoptotic form [150]. Similarly, BID possesses at least three splice variants, one of which (BID(S)) abrogates the pro-apoptotic effects of activated BID [151]. PUMA also has four potential splice variants although only PUMA-α and PUMA-β have the BH3 domain and their activity is thought to be similar [117]. BAX also possesses splicing variants that may serve different functions, although these remain unclear [152].

1.2.3.2.6. Post-Translational Control of BCL-2 Family Members

In addition to transcriptional control and alternative splicing, post-translational modifications play an important role in modifying the activity of BCL-2-family members. Among others, these modifications include proteolytic cleavage, phosphorylation, and ubiqutinylation. A prime example of proteolytic cleavage is the processing of BID into its truncated short form tBID (or p15). This process exposes the BH3 domain of BID, which is thought to allow its interaction with BAX [139]. This process is often catalyzed by caspase 8, which can be notably activated following membrane receptor signaling (e.g. Fas receptor) [138, 153]. However, as tBID lacks a hydrophobic region, it requires further addition of a mirystoyl group to integrate the mitochondrial outer membrane [154].

Many BH3-only proteins can be phosphorylated in response to cellular stress or to cytokines. One of the classic examples is BAD, that is maintained in a phosphorylated state by the persistent activity of kinases such as Akt [155]. In its phosphorylated state, BAD binds to 14-3-3 proteins that sequester it and prevent its action [156]. BAD must be de-phosphorylated by phosphatases such as calcineurin [157] in order to be activated. BIK/NBK can also be phosphorylated by kinases such as casein kinase II (CKII), although in contrast with BAD,

phosphorylation of BIK/NBK leads to its activation [158]. Similarly, BIM and BMF can be phosphorylated by JNK on dynein light chain binding motifs, which releases them from the cytoskeleton in response to microtubule-targeting agents such as Taxol (BIM) or resulting from anoikis (BMF) [159]. On the other hand, ERK-mediated phosphorylation of BIM promotes its ubiquitination and proteasome-mediated degradation and this event is prevented upon EnR stress by the action of the PP2A phosphatase [149]. Although this has not been particularly well studied to date, other BCL-2-family members can be targeted for degradation by the ubiquitin/proteasome system. One well-known example is MCL-1, which contains a PEST domain that targets it for degradation by the proteasome following poly-ubiquitination by the Mule/ARF-BP1 E3 ubiqutin ligase [38]. This event can be blocked by phosphorylation of the PEST sequence by ERK signaling [160].

1.2.3.3. Effectors of Apoptosis

Once the integrity of mitochondrial outer membrane has been fully compromised, various effectors of apoptosis are released from the inter membrane space. These proteins are responsible for producing the hallmark morphological changes associated with apoptotic cell death. With respect to this, caspases play a crucial role.

1.2.3.3.1 Caspases

Caspases are selective cysteine-proteases that cleave their substrate following an aspartate residue [161]. There are at least 11 caspases expressed in humans, initially produced as inactive zymogens (pro-caspases) (207-210). Active caspases are hetero-dimers that result from the proteolytic processing of the pro-caspase form which yields a short C-terminal fragment, a long middle fragment and an N-terminal pro-domain that does not participate in hetero-dimerization. However, when fully activated, caspases are in fact composed of two hetero-dimers. Notably, active caspases can activate their own inactive zymogens as well

as that of other caspases, yielding what is known as the caspase cascade [162-166].

There are three essential categories of caspases: intitiator caspases, effector caspases, and caspases involved in inflammatory cytokine maturation. Of these, inititator and effector caspsases are of particular importance for apoptosis. Initiator caspases include caspase-2, -8,-9 and -10 while effector caspases consist of caspases -3, -6 and –7 [167, 168]. As one might gather from the nomenclature used to classify them, initiator caspases initiate the caspase cascade. As mentioned previously, caspases -2 and -9 are present in the inter-membrane space of the mitochondrion (see section 1.2.3.1.3). Their release from the mitochondria in response to cellular alterations that tip the balance towards the activation of pro-apoptotic BCL-2-family members initiates what is termed the intrinsic apoptotic pathway [62, 89, 92]. The role of the initiator caspases is to activate the effector caspases, which mediate the proteolysis of many substrates that lead to the hallmarks of apoptosis. One of the principle modes of activation of the effector caspases via the intrinsic pathway is through what is called the apoptosome, a complex containing Apaf-1 and pro-caspase 9 that forms in the presence of cytochrome c in an ATP-dependent fashion [169-172]. Caspase-2 has also been reported to form similar complexes with RAIDD and PIDD, which has been named the PIDDosome. The formation of the apoptosome (and/or the PIDDosome) leads to auto-activation of caspase-9 (or caspase-2), which in turn activates caspases -3, -6 and –7 [173-176]. In contrast, caspases -8 and -10 are associated to the extrinsic or death receptor pathway. This pathway is essentially associated to the TNF death receptor family that includes Fas, TNF-R1, DR3, Trail-R1/DR-4, DR5/Killer/Trail-R2 and DR6 [177]. Upon the cell surface receptor binding of death-inducing ligands such as FasL, Trail and various others, the death receptors oligomerize and recruit adaptor proteins such as FADD or TRADD via interactions through the death domain (DD). The adaptors subsequently recruit initiator caspases -8 and/or -10 forming what is called the Death Induced Signaling Complex (DISC). Once in close proximity, caspases -8 and caspase-10 undergo auto-activation and may initiate the caspase cascade. In

addition, as caspase-8 can cleave BID into its active from tBID, the intrinsic pathway can feed back to the mitochondrial pathway, accelerating to apoptotic process [178-181].

1.2.3.3.2 Caspase Targets

Once the effector caspases are activated, they are free to cleave key substrates leading to DNA fragmentation and the formation of contained apoptotic bodies. One of these is I^{CAD}, a natural inhibitor of CAD (caspase activated DNAse). Cleavage of I^{CAD} leads to activation of CAD and subsequent DNA fragmentation [182, 183]. Effector caspases also lead to the cleavage of proteins such as PARP [184] and DNA-PK [185] that are involved in the maintenance of genome integrity. In addition, the constitutively active truncated form of ROCK-1 resulting from caspase-mediated proteolysis leads to membrane blebbing through its role in actin cytoskeleton contraction [186].

1.2.3.3.3. Inhibitors of Apoptosis

The activity of the caspases can be inhibited by a class of proteins called IAPs (inhibitors of apoptosis). These proteins, including cIAP1, cIAP2, xIAP, ML-IAP, Survivin and Apollon, inactivate caspases through interactions via their BIR domains (baculoviral IAP repeat) [187-192]. Interestingly, some of these proteins, such as cIAP1, xIAP and Apollon contain Ubiquitin ligase RING domains and can mediate their own targeting for proteasomal degradation in response to an apoptotic stimulus [193, 194]. As mentioned previously, the activity of IAPs can be inhibited subsequently to leakage of the mitochondrial inter-membrane space through the action Diablo/Smac and Omi/HtrA2 (see section).

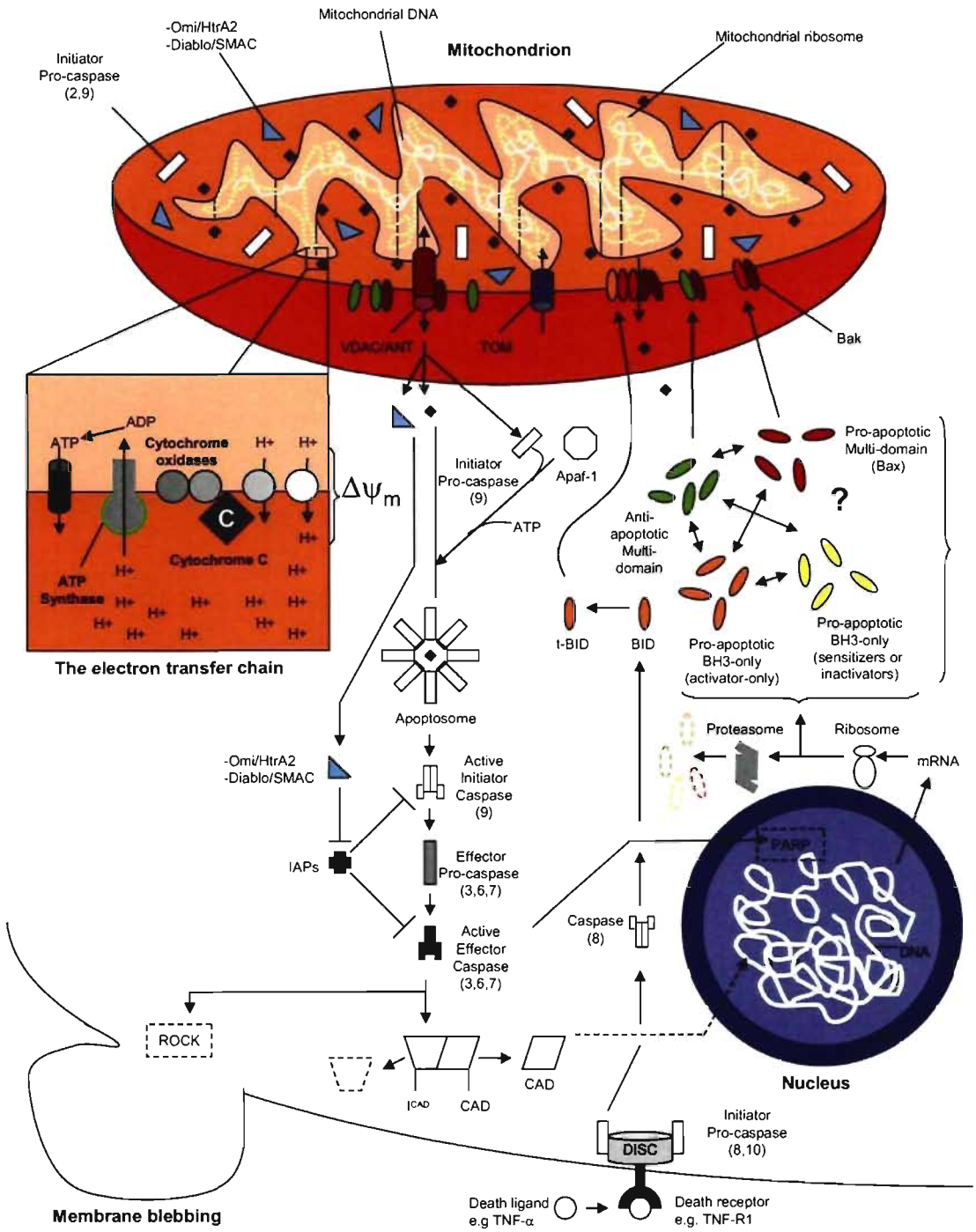

Figure 2. Overview of mitochondrial functions and intrinsic/extrinsic pathways of apoptosis. Refer to sections 1.2.3 through 1.2.3.4. Dotted lines indicate degradation events while lines ending in bars indicate inhibitory effects

1.2.3.4. Non-Apoptotic Cell Death

It is important to mention that although bone fide apoptosis requires caspase activation in order to occur, cell death does not necessarily require the full completion of the apoptotic program. As was mentioned previously, the formation of the apoptosome is an ATP-dependent process and as such, it is thought that metabolic shutdown leading to rapid energy depletion can prevent the execution of the normal apoptosis program, leading instead to necrosis or some intermediate form of death in between (e.g necrapoptosis [195]). In addition, preventing caspase activation using chemical pan-caspase inhibitors such as ZVAD-fmk [196] does not always protect from cell death but may instead prevent the appearance of apoptotic hallmarks such as membrane blebbing and cell shrinkage. These modes of death can be termed caspase-independent, as they do not require the activity of caspases in order to occur, even though caspase activity and some of its associated phenotypical manifestations may be observed throughout the process [197]. Another form of cell death that can occur in absence of caspases is mitotic catastrophe, which occurs during mitosis and is characterized by poly-nucleation [198]. To a certain extent, irreversible growth arrest (senescence) can also be viewed as a form of caspase-independent cell death even though the metabolic activity of these senescent cells can persist for some time [199].

1.2.4. Deregulation of the Cell Cycle and Apoptosis in Cancer

Having reviewed the various processes of cellular homeostasis, we are now ready to explore how these are fundamentally altered in the context of the cancer cell. Before we proceed to address this in the specific case of prostate cancer (see the following section), we will briefly review important concepts and key molecular pathways implicated in cancer development.

1.2.4.1 Important Conditions for the Development of Cancer

It is thought by some of the pioneers in cancer research that specific characteristics need to be acquired by cancer cells in order to attain full-fledged

malignancy, although these conditions may be attained using several strategies [200]. It has been suggested that cancer cells must become self sufficient in growth signaling and acquire limitless replicative potential, become refractory to growth inhibition signals and able to evade apoptosis, acquire the ability to sustain angiogenesis and develop the ability to invade surrounding tissues and metastasize. As a last condition, it is thought that genomic instability can be a catalyst for the rapid acquisition of these abilities. Notably, at least three of these characteristics implicate the inadequate regulation of the cell cycle and of apoptosis.

1.2.4.1 Self-Sufficiency in Growth Signaling.

As mentioned previously (sections 1.2.1-1.2.2), the progression through the cell cycle is strongly influenced by growth factor signaling, particularly at the G1 phase through the up-regulation of D-type cyclin expression. As such, in order to continually divide, cancer cells must acquire the ability to reproduce growth factor signaling in the absence thereof. Although this can be achieved in many ways, a few key pathways are frequently de-regulated in cancer cells.

1.2.4.1.1. Receptor Tyrosine Kinases and the Ras Pathway

Receptor tyrosine kinases play a major role in growth factor signaling. This class of receptors accommodates a wide range of growth factor ligands including transforming growth factor α (TGF-α), platelet-derived growth factors (PDGF), insulin growth factors (IGF) and epidermal growth factors (EGF) [201-203]. Receptor tyrosine kinase (RTK) signaling proceeds through two major pathways whose activation depends on the ligand and receptor involved. The first pathway involves Ras and its downstream signaling effectors, which include Raf, MEK and MAPKs and leads to the phosphorylation of various proteins, including the Myc transcription factor that is involved in cell cycle progression, among others, through the regulation of cyclins [204-206]. It is thought that roughly 25% of all human cancers express a mutated form of Ras that is active independently of receptor tyrosine kinase signaling [206]. In addition, many cancers exhibit elevated levels of RTKs such as EGFR or Her-2 [207, 208], as well as increased

autocrine production of RTK ligands such as TGF-α [209]. Alternately, augmented expression of downstream effectors of Ras signaling, particularly of Myc, is observed in cancer (particularly in Burkitt's lymphoma) [210] and over-expression of Myc drives tumor development in several mouse models [211] (for an overview, see Fig. 1.3).

1.2.4.1.2. The PI3K/Akt Pathway

Another pathway important for self-sufficiency in growth factor signaling involves PI3K and its downstream effector Akt. PI3K activation leads to the phosphorylation of membrane-bound phosphatidyl-inostol (4,5) bi-phosphate (PIP2) into phosphatidyl-inosoitol (3,4,5) tri-phosphate (PIP3). The conversion of PIP3 back to PIP2 is catalyzed by the PTEN phosphatase, which acts as an important negative regulator of this pathway and is often mutated in cancer (see section). Membrane-bound PIP3 activates phosphatidylinositol-dependent kinases (e.g. PDK1), which leads to the phosphorylation and activation of Akt [212]. Counterbalancing the action of PDKs, the PP2A phosphatase can de-phosphorylate and inactivate Akt [213]. Notably, Akt is a central signaling hub as it sits at the junction of several signaling pathways [214]. Akt activation also leads to the phosphorylation and activation of mTOR, which stimulates protein translation and consequently affects several other pathways [215, 216] (see section). As mentioned previously, active Akt also maintains the BCL-2-family member BAD in a phosphorylated and inactive 14-3-3 protein-bound state, which promotes cell-survival [217]. Finally, Akt can also activate the NF-κB pathway [218, 219] (for an overview, see Fig. 1.3).

1.2.4.1.3. The NF-κB Pathway

NF-κB is a dimeric protein composed of combinations of NF-κB monomer subunits p50, p65 (RelA), p52, RelB and c-Rel. NF-κB activity is directly regulated by the action of IκB proteins (e.g. IκB-α, β, ε, p100) and IκB kinases (IKK-α, β, γ, ε) [49, 220, 221]. IκBs retain NF-κB in the cell cytoplasm and diminish its transactivation potential whereas IκB kinases phopsphorylate

both IκBs as well as NF-κB itself. Phosphorylation of IκBs leads to their proteasome-mediated degradation and leads to the release and nuclear translocation of NF-κB trancription factors. In addition, phosphorylation of NF-κB subunits (eg. p65) can lead to increased transactivation potential [222]. In what has been termed the classic or canonical NF-κB pathway, activation of IκB kinase β (IKK-β) by stimuli such as TNF-α, leads to the phosphorylation of IκB-α. Phosphorylated IκB-α is quickly targeted for proteasome-mediated degradation through the action of $SCF^{\beta TrCP}$ [34]. Degradation of IκB-α is thought to lead to the specific release of p50 and p65 homo-and hetero-dimers. In the non-classical or non-canonical pathway, IKK-α leads to the phosphorylation and proteasome-mediated proteolytic processing of p100 into the NF-κB p52 subunit. Dimers formed from RelB and p52 as well as p52 homo-dimers are generally associated to this pathway [34, 49, 223] (for an overview, see Chapter I Figure 3). The non-classical pathway is typically activated by stimuli such as lymphotoxin B and lipopolysaccharide (LPS) [224] but also following long-term androgen stimulation as discovered recently in LNCaP prostate cancer cells [225]. By binding κB sequences within gene regulatory elements, NF-κB controls the

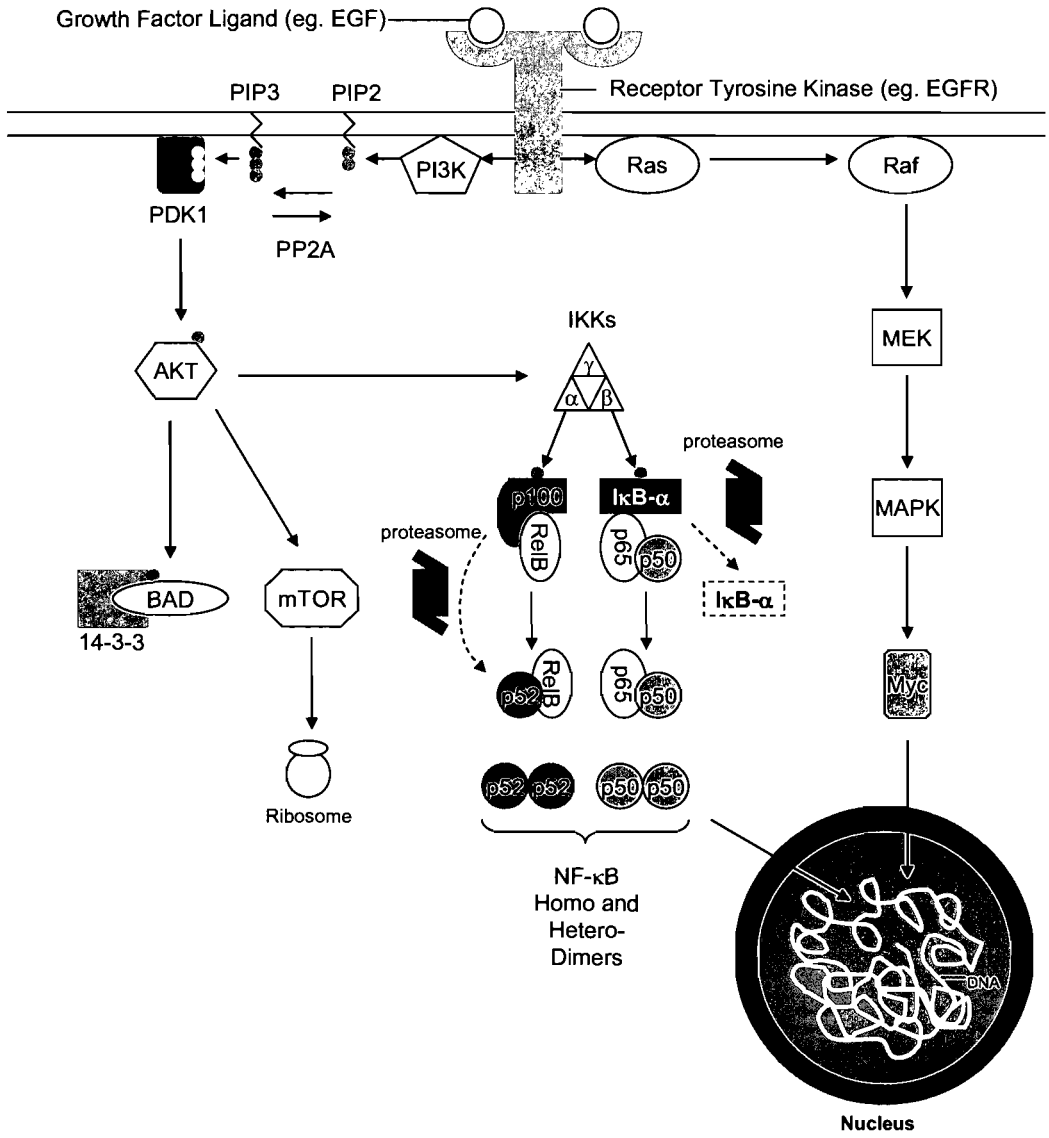

Figure 3. The Receptor Tyrosine Kinase, Ras, P13K/Akt, and NF-κB pathways. Refer to sections 1.2.4.1.1 through sections 1.2.4.1.3. Green circles indicate a phosphorylation event whereas dashed lines indicate a degradation event.

expression of hundreds of genes, many of which are involved in cell proliferation and cell survival [223].

1.2.4.1.4. Alternate Pathways: from Wnt to Nuclear Receptors

In addition to the frequently observed constitutive activation of the Ras and PI3K signaling axes, cancer cells can exhibit aberrant signaling in the Wnt pathway, involving its receptor (e.g Frizzled) and effectors Disheveled, GSK-3β, APC and β-catenin [226]. Increased signaling through G-protein coupled receptor and its effectors adenylate cyclase, PKA and the CREB transcription factor are also observed in cancer cells [227]. Furthermore, cancer cells can over-express and secrete interleukin-family cytokines such as IL-3 and IL-6 [228, 229], whose autocrine effects are mediated by interleukin receptors (e.g gp130) that signal through JAK and STAT trancription factors [230, 231]. Finally, nuclear receptor family transcription factors, which act as direct sensors and transducers of signaling by various ligands including steroid hormones, are often abnormally active in cancer. This event is frequently observed in breast cancer for the Estrogen Receptor (ER) [232] and in prostate cancer (PCa) for the androgen receptor (discussed in further detail in section 1.3.5).

1.2.4.2. Insensitivity to Growth Inhibitory Signals

1.2.4.2.1 Pocket Proteins and Cell Cycle Inhibitors

In addition to mimicking persistent signaling from growth factor signaling components, the cancer cell must become refractory to signals that promote quiescence through cell cycle exit and/or differentiation. Importantly, many of these signals converge to the pocket protein family members pRb, p107 and p130 [47, 233]. Particularly loss of pRb functionality is frequently observed in cancer and several oncogenic viral proteins such as E1A and E7 also inactivate Rb [233, 234]. Among others, loss of pocket proteins, particularly pRb, leads to the constitutive activity of E2F transcription factors, which promotes cell cycle progression in a manner that cannot be halted by anti-growth signals, such as that provided by TGF-β signaling for example [235]. Notably, although some of the

anti-proliferative effects of TGF-β are mediated by the phosphorylation pRB, TGF-β can also reduce the expression of myc and up-regulate CDK inhibitors such as p15 (INK4b), $p21^{Cip1}$ and $p27^{Kip1}$ through the action of SMAD transcription factors [235, 236]. As such, de-regulation of these proteins (e.g up-regulation of myc or loss of CDK inhibitors) can lead the cell to become unresponsiveness to growth-inhibitory signals.

1.2.4.2.2. p53 and the Response to DNA Damage

Growth inhibitory signals can also originate from internal cell signals such as those coming from DNA damage. In response to DNA damage, signaling kinases such as ATM and ATR activate a multitude of targets including p53, Bid and IκB-α [237, 238]. In this pathway, p53 is particularly important because it leads to the up-regulation of genes involved in cell cycle arrest such as $p21^{Cip1}$ as well as genes involved in apoptosis such as NOXA, PUMA and BAX [53, 117, 118, 239]. As such, p53 acts as a gatekeeper for the integrity of the genome that can be compromised in response to various genotoxins but also through un-controlled progression through the cell cycle via the aberrant activation of oncogenes such as Ras for example [240]. Likely due to this central position in the cell, p53 is mutated in over 50% of cancers [241]. Notably, a negative regulator of p53, the MDM2 ubiquitin ligase, is also overexpressed in roughly 10% of human cancers [242]. In parallel, the INK4B locus-encoded $p16^{INK4B}$ (CDK inhibitor) and $p19^{ARF}$, a negative regulator of MDM2, are frequently lost in cancerous cells [243]. In any case, without p53, cancer cells are thought to be free to progress through the cell cycle and accumulate mutations that may lead them to even more rapid acquisition of additional characteristics important for optimal malignancy [200]. However, as nearly half of human cancers are not mutated for p53, it is clear that p53-mediated functions such as apoptosis, cell-cycle control, and DNA repair can be bypassed by other means as well.

1.2.4.3. Resistance to Apoptosis

Another fundamental characteristic of cancer cells is their ability to bypass both extrinsic and intrinsic death signals. Similarly to the many ways that can be employed by cancer cells to attain growth factor cell-autonomy, resistance to apoptosis can be achieved by a variety of alterations in the pathways that lead to apoptosis. In fact, some of the signaling pathways that lead to growth factor self-sufficiency and resistance to growth inhibitory signals also lead to resistance to apoptosis. For example, within the PI3K pathway, over-activation of Akt leads to the inhibition of the pro-apoptotic protein BAD. Still within this pathway, the activation of NF-κB can lead to the up-regulation of anti-apoptotic proteins such as BCL-2, BCL-XL and A1/Bfl [142-144, 244] as well as of IAPs such as xIAP [245], c-IAP1 and c-IAP2 [246]. Notably, the prototypical BCL-2 family member (BCL-2) was first discovered in the context of B-cell lymphoma (hence its name) where it is up-regulated due to a chromosomal translocation that subjects it to transcriptional control by the immunoglobulin locus [247].

The down-regulation or loss of pro-apoptotic proteins may also promote cancer progression. However, because the functions of some pro-apoptotic proteins appear to be highly redundant, their contribution to tumor progression may be fairly context specific and it is likely that the inactivation of many pro-apoptotic proteins is required to achieve a maximal impact [248]. For example, Bax-null transgenic mice very infrequently develop spontaneous tumors [249]. Nonetheless, some pro-apoptotic proteins have been found to increase tumorigenesis in mouse models, particularly in combination with oncogenes that stimulate cell proliferation. For example, loss of one or both alleles of Bim in transgenic mice expressing a myc transgene accelerates the onset of acute B-cell leukemia [250]. Analogous results are obtained in a similar model but where mice are instead deficient in BAX [251]. In addition, mice completely deficient for BID develop chronic monomyelocytic leukemia after approximately 2 years of life [252]. Although this is not always be the case as we will see in chapters II and V, loss of p53 curtails the up-regulation of its pro-apoptotic targets NOXA, PUMA and BAX in response to DNA damage and cytotoxic drugs [239, 253]. This phenomenon has been hypothesized to contribute to tumorigenesis in p53

defficient tumors. However, supporting the hypothesis that the cancer cell may require the inactivation of many pro-apoptotic genes to promote tumorigenesis, mice deficient for NOXA only modestly co-operate with transforming oncogenes E1A and Ras, which is aided by the additional loss of Bax [254]. On the other hand, mouse embryonic fibroblasts expressing E1A and Ras in which PUMA has been down regulated via shRNA exhibit markedly enhanced tumor formation when implanted in immuno-compromised mice [255]. These observations also appear to correlate with the relative contribution of PUMA and NOXA to p53-mediated apoptosis in several contexts [248].

1.2.4.4 Other Important Characteristics.

In addition to the cancer cell characteristics involving the cell cycle and apoptosis described in section 1.2.4.1 through 1.2.4.3, it is worthwhile to briefly mention key pathways that can confer limitless replicative potential, sustained angiogenesis and increased invasiveness.

1.2.4.4.1. Unlimited Replication

In contrast with normal cells that can only replicate a fixed number of times (60-70 divisions), cancer cells have unlimited replication potential and are as such considered immortal. One of the processes that have been implicated in this phenomenon is the de-regulated increase of telomere length. Telomeres are formed from highly repetitive sequences present at the ends of each chromosome and prevent the loss of genetic information at the chromosome ends following replication. The length of telomeres is normally maintained by enzymes such as Telomerase or through a homologous recombination based pathway termed ALT or alternative lengthening of telomeres. Hence, de-regulated expression of Telomerase or of the enzymes involved in ALT can lead to increased telomere length [9, 256-260]. In addition, as the maintenance of telomere length is highly influenced by chromatin compaction, altered activity of proteins such HATS, HDACs and histone methylases (e.g SUV39H1 and SUV39H2) can also impact the length of telomeres [9].

1.2.4.4.2. Sustained Angiogenesis

In maintaining access to blood and oxygen, the VEGF growth factor is thought to be of major importance as it is one of the main signaling molecules involved in the recruitment of peripheral blood vessels. By producing cytokines such as VEGF either directly or indirectly, growing tumors can re-route blood vessels in order to remain well-oxygenated [261]. Alternatively, cancer cells that lay in the center of tumors where hypoxic conditions are encountered may constitutively up-regulate HIF-1α, a transcription factor that promotes the production of TGF-α, PDGF and VEGF in acute low-oxygen conditions [262]. Under normal conditions, HIF-1α is degraded by the proteasome through the action of the pVHL ubiquitin ligase, which is notably frequently mutated or lost in some forms of cancer (eg. renal cancer, hemangioblastoma) [263, 264]. In addition, HIF-1α is stabilized and up regulated by aberrant activation of the mTOR pathway [265, 266].

1.2.4.4.3. Enhanced Invasiveness

The ability of the cancer cell to invade the surrounding tissue and infiltrate distant sites can be achieved by various means. Notably, matrix metaloproteinases (MMPs) such as MMP-9 are thought to play a role in this process [267, 268]. In addition, cell adhesion molecules including integrins and E-cadherin can play a role in the process of cancer cell detachment form the primary tumor site and attachment to distant sites. Notably, these proteins are also involved in cell signaling, such as E-cadherin within the β-catenin pathway, which can be important for cell survival in conditions of anoikis (cell detachment) that may otherwise lead to apoptosis [200, 269].

1.3. Prostate Cancer Development and Pathology

Now that we understand the over-arching principles implicated in cancer development, we can begin to look at how cancer develops in the specific context of the prostate. Before we can fully appreciate this disease however, it is

important to briefly review some of the basic anatomical characteristics of the prostate in addition to the known factors that regulate its development.

1.3.1 Anatomy of the prostate

The prostate is a primarily exocrine gland situated at the base neck of the bladder and in front of the rectum. In adults, the normal prostate weighs roughly 15-20 grams although prostate size often increases with age [270]. The prostate is composed of two lobes that surround the proximal part of the urethra extending from the bladder neck [271]. Within the prostate, there are three histologically distinct regions, discernable on the basis of the glandular architecture. The peripheral zone represents roughly 70% of the prostate, while the central and transitional zones represent approximately 25% and 5% of the prostate volume respectively [272]. The function of the prostate is to secrete a nutrient-rich fluid that also contains enzymes involved in sperm liquefaction such as prostate specific antigen (PSA) [270, 273, 274]. The alveolar architecture of the prostate gland allows for the collection of the prostatic fluid into the urethra, where it rejoins secretions from the seminal vesicles, which are situated above the prostate between the bladder and the rectum [270, 271].

1.3.1.1 Cellular composition of the secretory glands

The secretory glands that constitute the glandular network are composed of three cell types. The luminal epithelial cells normally compose a single layer of differentiated cells at the lumen interface and are the main contributors to the secreted prostatic fluid [275]. Surrounding the layer of differentiated luminal cells is a much thinner layer of basal cells, which are essentially the renewable progenitors of both luminal cells and neuroendocrine cells [276]. Neuroendocrine cells produce neuropeptides involved among others in the control of cellular proliferation [277]. These cell types can be differentiated by the expression of specific proteins. Notably, whereas only luminal cells express the AR and cytokeratins 8/18, cytokeratin 5 and cytokeratin 34βE12 are primarily expressed in basal cells [276]. Finally, the prostate secretory glands are surrounded by fibro-muscular tissue referred to as the stroma, which is thought to play a role in the

regulation of epithelial cell growth through the secretion of growth promoting factors such as FGF as well as growth inhibitory factors such as TGF-β[278-280].

1.3.2. Factors Important for Prostate Development

The development of the prostate is strongly influenced by androgen growth factors. The testes are the main producers of androgens, although the adrenal glands also produce a small amount. The testicular production of androgens is regulated by the LHRH (lutheinizing hormone-releasing hormone) sex hormone that is secreted by the pituitary gland [281-284]. The androgen thought to be the most active throughout prostate development is dihydrotestosterone (DHT). Notably, one important enzyme involved in the production of DHT is 5-α-reductase, which catalyses the conversion of the double bond present in position 4,5 of testosterone into a single bond through the addition of two hydrogens [282]. DHT and androgens in general mediate their effects through the androgen receptor (AR), a nuclear receptor family transcription factor (described in greater detail in section 1.3.5).

Although the role of androgens in prostate development is clear, little is known about other factors that influence prostate development. In fact, many of the genes currently thought to regulate prostate development are regulated by androgens. For example, androgens regulate the expression of the NKX3.1 homeobox transcription factor, thought to play an important role in prostate development [285, 286]. The FGF-10 cytokine, which plays a role in establishing glandular branching is also regulated by androgens [287]. In addition, a very recent study has further established a role for androgens in up-regulating the expression of the FGF-10 receptor FGFRiiib, of Sonic hedgehog and its receptor Ptc, of Hoxb13, and of BMP7, all factors that are thought to contribute to prostate development and morphogenesis [288].

1.3.3. Prostate Cancer: Origins, Prevalence, and Risk Factors

Prostate cancer typically develops from the peripheral zone of the prostate, accounting for 60-70% of prostate cancers (PCa) [289]. PCa tumors arise almost exclusively from the epithelial cells that compose the prostate glandular network. Furthermore, PCa develops most frequently from luminal and basal cells whereas tumors of neuroedocrine origin are more rare [290].

According to recent statistics, 22 300 new cases of prostate cancer are expected for 2007 in Canada, making it the most frequently diagnosed cancer. However, in terms of mortality PCa ranks as the third most frequent killer in men following lung and colorectal cancer, with an expected 4300 deaths in Canada for 2007 [291] .

Although several studies have set out to understand the etiology of PCa, only a few risk factors have been established so far. Age is thought to be one of the most important factors. Notably, as suggested by a recent report from the Canadian Cancer Society, the 10-year risk for PCa development was found to be nearly 70 times higher in 70-79 year old men as compared to men age 40-49 [292].

It is thought that only 5-10% of PCa cases are linked to hereditary causes. Notably, PCa risk increases when a close family member such as a brother or father has developed the disease [293]. On the other hand, the specific causes of hereditary PCa are far from clear even though a few potential polymorphisms and susceptibility genes have been identified. These include genes such as endoribonucleases RNAseL [294] and ELAC2 [295], which may respectively interfere with antiviral response [296] and anti-proliferative TGF-β signaling [297], NBS1, CHEK2 [298, 299], and $p27^{Kip1}$ that are involved in cell cycle control, as well as BRCA1 and BRCA2 that are involved in DNA repair [300, 301]. In addition certain polymorphisms of the AR (see section 1.3.5) and of 5-α-reductase have been found to be associated to PCa risk [302].

Although isolating the specific dietary components involved is often particularly challenging due to a multitude of potentially confounding factors, it is becoming increasingly clear that nutritional habits are linked to the progression of

PCa or lack thereof. Nutrients currently thought to have a protective effect against PCa include lycopenes (such as found in tomatoes) [303], β-carotenoids [304], vitamin D [305], vitamin E [306], selenium (as found in garlic) [307], sulforaphane, indole-3 carbinol (in cruciferous vegetables such as broccoli) [308], polyphenols (green tea, red wine) [309, 310], and curcumin (Indo-Asian spice) [311]. Other foods such as soy are rich in factors thought to protect against PCa including isoflavones such as genistein, daidzein and equol [312] as well as other molecules such as inositol hekakisphosphate (as we will review in further detail in section 1.4.4). In contrast, high dietary calcium and zinc intake may potentially increase PCa risk [313].

Likely linked to both nutritional and genetic factors, race has emerged as another risk factor for PCa. Notably, African Americans are the most susceptible to PCa, followed by Caucasians, Hispanics, and Asians [314].

1.3.4. Genetic and Molecular Events Involved in Prostate Cancer Progression

Whereas hereditary PCa constitutes a minority of PCa cases, sporadic genetic events are thought to account for over 90% of the observed cases. In the context of cellular homeostasis, it is therefore relevant to point out some of the commonly encountered chromosomal alterations in PCa and their impact on the process of prostate epithelial cell transformation.

1.3.4.1. Chromosomal Losses and the Role of Tumor Supressors

Partial or complete loss of chromosomes 5q, 6q, 8p, 9q, 10p, 10q, 13q, 16q and 17p has been detected to various extents in PCa tumors [315-334]. Although the mediators of the effects on tumorigenenesis present within the chromosomal domains have not all been identified, it is reasonable to expect that they involve various tumor repressors. Of note, chromosome 9q contains the CDK inhibitors p15 (INK4B) and p16 (INK4A) [316] (see section 1.2.1.3.). As mentioned previously, the IN4KA locus also codes for p19 (ARF), a negative regulator of the MDM2 protein that targets p53 for degradation (see section

1.2.4.2.2.). Chromosome 10q contains the PTEN phosphatase, a negative regulator of the PI3K pathway (see section 1.2.4.1.2) and is lost in up to 63% of metastatic PCa [317, 330]. In addition, transgenic mice that present homozygous loss of PTEN specifically in the prostate progressively develop metastatic PCa [335]. Chromosome 13q contains pRb involved in cell cycle progression (see sections 1.2.1.3 and 1.2.4.2.1), which is lost in approximately a third of localized PCa and in up to 75% of metastatic PCa [315, 317]. Chromosome 16q contains the cell adhesion molecule E-cadherin, which is also involved in regulating β-catenin signaling [317, 325, 332]. Finally, the gene encoding for the p53 protein involved in response to DNA damage, genome integrity, and apoptosis, is located on chromosome 17p that is lost in 18% of localized PCa and up to 50% of metastatic prostate tumors [317, 334].

1.3.4.2. Chromosomal Gains and the Role of Oncogenes

In parallel, chromosomal gains and gene amplifications are also observed in PCa tumors. Notably, gains in chromosomes 1q, 2q, 7p, 8q, 11p and 17q have been reported [317, 318, 336-339]. The effects of chromosome 8p gain, a fairly frequent event (observed in up to 85% of metastatic PCa), are thought to be associated to the myc gene that it harbors [317, 339, 340] (see section 1.2.4.1.1). The contribution of myc to locally invasive PCa has been also observed in transgenic mice that over-express myc in a prostate-specific fashion [341]. Although less frequent (8%), the effects of chromosome 7p gain are attributed to EGFR [337] (see section 1.2.4.1.1). Similarly, the EGFR homolog HER-2/Neu/ErbB2 is present on chromosome 17q whose gain was reported in 30% of metastatic tumors [317, 339]. Finally, the gain of chromosome 11p, observed in nearly half of metastatic PCa, is thought to promote tumorigenesis in part via increased expression the Ras oncogene [317].

1.3.4.3. Other Important Genetic Events

Beyond major chromosomal alterations, some specific genetic transformations are thought to be involved early on in PCa development. Notably,

the role of the NKX3.1 gene was established through the observation that NKX3.1 knockout mice develop epithelial hyperplasia and dysplasia [342], Besides its role in the morphogenesis of the prostate and associated role in cell differentiation, it is interesting to note the NKX3.1 has recently been found to negatively regulate AR expression, stabilize p53, and inhibit Akt activation in PTEN knockout mice [343]. Another potentially important recent discovery was that of the TMRPSS2-ETS gene fusions that result from chromosomal rearrangement and that have been detected in almost 80% of PCa tumors. These fusions bring together the androgen-responsive gene promoter of the TMRPSS2 gene in proximity with ETS transcription factors, which promote cell proliferation [344]. Last but not least, and indirectly impacting other potential oncogenes such as the TMRPSS2-ETS gene fusions, several mutations, gene amplifications and X-chromosome alterations link the AR to PCa progression. Notably, transgenic mice over-expressing the AR specifically in the prostate exhibit increased cell proliferation and signs of the initial stages of invasive PCa [345].

1.3.5. The Androgen Receptor

One of the most significant advances in PCa treatment in the last century was linked to the discovery that castration (loss of androgen production) leads to the remission of metastatic prostate tumors [346] (see section 1.3.7.3). These experiments established androgens and their cellular target the AR as crucial regulators of prostate and PCa cell survival and proliferation.

As mentioned previously, the AR is a member of the large family of nuclear receptor transcription factors. Its major structural components include an N-terminal transactivation domain (AF-1), a central DNA-binding domain (DBD), and a hinge region that links the DNA-binding domain to a second C-terminal trans-activation domain (AF-2), within which is nested the androgen-binding domain (ABD) [347].

1.3.5.1. The AF-1 Domain

The AF-1 domain is particularly important for AR-mediated transcription. This is brought to light by the observation that deleting the ABD domain of the AR yields a receptor with constitutive transcriptional activity, equivalent to the full-length receptor [348]. The AF-1 domain interacts with a variety of well known transcriptional co-activator proteins including ARA family co-activators (ARA24/RAN[349], ARA70[350], ARA160[351], ARA 267-α[352]), SRC-1[353], CBP[354] and TFIIH[355]. Some cell cycle proteins, including cyclin E [356] and pRb [357], also interact with the AF-1 domain and lead to enhanced AR-mediated transcription. The AF-1 domain interacts with several other proteins with various functions such as BRCA1 [358], Raf [359], GAPDH [360], Caveolin-1 [361] and mitochondrial COXVb [362]; however, the biological significance of these interactions is less clear. The AF-1 domain contains both a poly-glutamine tract and a poly-glycine tract that are highly polymorphic due to polymerase slippage caused by the repetitive DNA sequence [363]. Notably, the poly-glutamine tract is thought to be important for co-activator recruitment [364]. Interestingly, the length of both poly-glutamine and poly-glycine tracts are linked to the risk of developing PCa [363].

1.3.5.2. The Hinge Region and the DNA-binding Domain

Whereas the hinge region is thought to play a role in the translocation of the AR towards the nucleus [348, 365], the DNA-binding domain mediates the interaction between the AR (which usually acts as a dimer) and androgen response elements (ARE) [364]. The DBD recognizes the AREs through two Zinc-finger domains that intercalate the DNA major groove [366]. Much like AF-1, the DBD/hinge interacts with various proteins including other transcription factors such as c-Jun [367], PDEF[368], ATF2[369], GR, OCT1/2 and p65(RelA) [370]. Contrary to the other transcription factors, the NF-κB subunit p65 (RelA) has an inhibitory effect on AR-mediated transcription. Analogously, other proteins such as calreticulin (a calcium-dependent chaperone) [371] and cyclin D1 [372] interact with the DBD/hinge and repress AR transcriptional activity.

1.3.5.3. The AF-2 and Androgen-Binding Domains

The AF-2 and the nested ABD mediate the binding of androgens and elicit androgen-dependent transcription. Overall, this combined domain interacts with several proteins including co-activators such as ARA54 [373], ARA55/Hic-5 [374] and the HAT named P/CAF [375]. The AF-2/ABD domain also interacts with other transcription factors such as β-catenin [376], which acts as a transcriptional co-activator, SMAD-3 [377] that acts as an AR co-regulator, and p53 that represses AR-mediated transcription [378]. Notably, the AF-2/ABD of the AR interacts with cytoplasmic regulatory chaperones and co-chaperones such as Hsp90 [379-381] and cdc37[382].

1.3.5.4. AR Phosphorylation

The AR is also known to interact with or be phosphorylated by several potein kinases. For example, Akt interacts with the AF-2/ABD domain [383] and phosphorylates the AR within AF-1 and AF-2, which is thought to prime the AR for poly-ubiqutination and proteasome degradation through the interaction of MDM2 with the AF-1 domain [384]. Other phosphorylation events mediated for example by PKA [385] and Src [386] onto the AF-1 domain or by JNK [387] towards the hinge region, can instead activate AR-mediated transcription. PKC signaling initiated by phorbol ester has also been reported to lead to phosphorylation on serine 650 of the hinge region. However, it remains unclear whether phosphorylation at this site influences AR transactivation [388, 389].

1.3.5.5. Effects of Androgen Binding

Importantly, the binding of androgens to the AR leads to structural rearrangements that lead to functional changes in the AR. When the AR is not bound by androgens, it is normally associated to chaperones such as Hsp90 and cdc37. Androgen binding disrupts the interaction between the AR and these cytoplasmic chaperones, which leads to the eventual shuttling of the AR towards the nucleus [348, 364, 365, 390]. In addition, androgen binding causes the displacement of α-helix 12 within the AF-2/ABD, which frees interaction sites important for co-activator recruitment [391, 392]. Bound by androgens, the AR has been reported to directly and indirectly regulate the expression of hundreds of

genes involved in a myriad of processes including differentiation metabolism, cell proliferation, cell cycle, and cell survival [393-395].

1.3.5.6. Cytoplasmic Androgen Receptor Functions

Interestingly, an increasing number of studies suggest that the AR may play additional roles in the cytoplasmic compartment, particularly at the level of the cytoplasmic membrane. One study done in osteoblasts and HeLa cells suggests that androgen-bound AR can trigger the MAPK signaling pathway prior to shuttling to the nucleus, by interacting with and activating Src via the AF-2 domain. This was associated to the resistance to apoptosis triggered by the DNA damaging drug etoposide [396]. Conversely, very recent studies done in PC3 prostate cancer cells suggest that, independently of androgens, the AR can inhibit EGFR signaling. This is thought to occur by preventing receptor internalization into endosomes via the caveolin-1/clathrin pathway [397], an event required for optimal EGFR signaling [398].

1.3.6. Commonly used Prostate Cancer Cell lines.

NKX3.1 and prostate-specific PTEN knockout mice as well as mice expressing the AR, myc (see sections 1.3.4.2 and 1.3.4.3) or transforming SV40 proteins [399] (see discussion section 6.3) specifically in the prostate are emerging as useful tools for the study of PCa development and treatment [400]. Although these recently developed mouse models have certainly contributed to our understanding of the factors important for PCa development, much of what is currently known stems from observations made from cell lines that were derived from either primary PCa tumors or metastases. This section will briefly review some important characteristics of the better known and most frequently used cell lines.

1.3.6.1. The DU145 Cell Line

DU145 cells were the first PCa cells to be isolated in culture. They were obtained from the brain metastasis of a 69-year old caucasian man that also had

lymphocytic leukemia [401]. The DU145 cell line contains on average 64 chromosomes and exhibits many chromosomal alterations. A mutant form of p53 as well as a non-functional mutant form of Rb is expressed in these cells [402, 403]. Interestingly, the DU145 may have originated from epithelial cells at a stage situated somewhere in between basal and luminal cell stage, as they do not express neuro-endocrine markers or the AR but simultaneously express cytokeratins 8, 18 and cytokeratin 5 [403-405]. The DU145 cell line forms aggressive tumors in immuno-compromised mice [406].

1.3.6.2. The PC3 Cell Line

Another cell line that is very frequently used is the PC3 cell line, which originates from the lumbar metastasis of a 62 year old Caucasian male [407]. The PC3 cell line is also fully aneuploid with an average of 58 chromosomes. Similarly to DU145 cells, PC3 do not express the AR although the do express cytokeratins 8, 18 and 5, suggesting they may originate from basal-luminal intermediates [402, 403]. Also analogously to DU145 cells, PC3 cells form metastatic tumors in mouse xenograft models. Notably, the p53 gene is mutated in PC3 cells, which results in a premature stop codon. As such p53 is not expressed in this cell line [403].

1.3.6.3. The 22Rv1 Cell Line

The 22Rv1 cell line was derived through serial sub-cutaneous xenografting of the parental androgen-sensitive cell line CWR22R in castrated mice, which was initially obtained from the primary prostate carcinoma of a patient in which the cancer had already metastasized to the bone [408]. Karyotype analyses of the 22RV1 genome have revealed that this cell line has an unstable genome and in particular, was found to have gained extra copies of chromosomes 7, 8, and 12 [402, 403]. Notably, as mentioned in section 1.3.4.2, chromosomes 7 and 8 are thought to promote tumorigenesis because they contain EGFR and Myc oncogenes respectively. In addition, the p53 gene of 22RV1 cells presents heterozygous mutations in exons 8 and 9 [403]. 22RV1 cells express cytokeratins 8 and 18 as well as the AR, although the AR presents two mutations, one in the

AF-1 domain and the other in the AF-2/ABD. 22Rv1 cell growth is stimulated by androgens although they continue to grow in absence of androgens [409], In addition, the growth of 22Rv1 cells is stimulated by EGF but and is not inhibited by TGF-β [403, 406]. 22Rv1 cells form large tumors in mice but do not metastasize when injected intra-prostatically [410].

1.3.6.4. The LNCaP Cell Line

The LNCaP cell line was isolated from a metastatic lymph node biopsy obtained from a 50-year old caucasian male [411]. LNCaP cells are aneuploid, reportedly containing an average of 76 to 91 chromosomes. In LNCaP cells, Chromosome 10q has suffered deletions in the PTEN locus and as such, LNCaP cells do not express PTEN. However, they express cytokeratins 8 and 18, a wild-type p53 and the AR [402, 403]. Notably, the AR is mutated at position 877 in the ABD which alters its specificity. Consequently, the LNCaP AR is responsive to a wider range of ligands, including flutamide, an anti-androgen used for androgen depletion therapy (see section 1.3.7.3) [412]. LNCaP cell growth is at least initially dependent on androgens; however, several LNCaP sublines have been derived from prolonged culture in androgen-depleted media [413]. When implanted in mice, LNCaP cells readily form tumors [406] but stay confined to the prostate when injected orthotopically [414].

1.3.6.5. RWPE-1 Cells

Unfortunately, there are very few human cell lines that have been derived from normal prostate epithelium and those that have been isolated are not well characterized. Of note the non-tumorigenic RWPE-1 cell-line was generated 10 years ago using human papillomavirus 18 [415] as an immortalizing agent. The RWPE-1 model is increasingly used as a model for carcinogenesis and cellular transformation, likely because un-modified RWPE-1 cells do not form tumors in mice [416, 417].

1.3.7. Detection, Pathology, and Current Treatment of Prostate Cancer.

1.3.7.1. Detection of Prostate Cancer

During the early stages of PCa when the tumor is relatively small and localized, the disease is largely asymptomatic. Although the digital rectal exam (DRE), which has been widely used for PCa detection during decades, can sometimes be sufficient to detect such tumors, its success rate is fairly poor at the early stages of PCa. The detection rate of early stage PCa was greatly increased subsequently to the discovery of PSA [418], an AR-regulated protein [419]. As mentioned previously, PSA is mostly a component of the prostatic fluid although small amounts are normally secreted in the blood stream. Increasing prostate size, increased AR activity, and importantly PCa tumor progression can all lead to an increase in PSA secretion in the blood. Detection of PSA in the bloodstream as a detection method was adopted towards the end of the 1980s and has proved to be one the most significant improvements in PCa detection to date [420].

1.3.7.2. Diagnosis and Histopathology

Unfortunately, bone fide PCa is not the only factor that can lead to an increase in PSA. For example, benign prostatic hyperplasia (BPH) can also lead to an increase in PSA [421]. As such, additional pathological information obtained from biopsies or trans-urethral resections of the prostate (TURP), a surgical procedure often used to re-establish urinary flow in patients with enlarged prostates due to BPH or PCa [422, 423], is often necessary to confirm the cancer diagnosis. At the tissue level, pathologists notably look at epithelial gland morphology patterns as well as the expression of cytokeratins to confirm or refute the presence of PCa. Glandular morphology is "quantified" according to the Gleason scale. This semi-subjective system is a 5-step scale based on the arrangement of the glands and the degree of differentiation of the epithelial tissue [424]. Pathologists then determine the Gleason score (2-10), which is the sum of the two major Gleason patterns (1-5). Importantly, a high Gleason grade/score is indicative of aggressive cancer [425, 426]. Cytokeratin expression is particularly useful for discerning between normal glands and what are considered to be pre-cancerous lesions, namely prostatic intra-epithelial neoplasia (PIN). Glands

considered as presenting PIN somewhat resemble normal glands but have several layers of luminal epithelial cells and have partially lost the basal cell layer, which can be detected by immunohistochemical staining for cytokeratin 5 [427] or cytokeratin 34βE12 [428].

1.3.7.3. Prostate Cancer Staging and Treatment

If the presence of PCa has been confirmed, its treatment will depend on the quantity of PSA in the blood and velocity of PSA progression, the Gleason score (when available), and the clinical stage [429]. The clinical stage (TNM) is determined from the available clinical information obtained by non-invasive means, such as the DRE and radiological imaging, pertaining to the extent of tumoral invasion and metastasis [429]. Notably, PCa metastases usually arise in the pelvic lymph nodes, the bones, and the brain. When it is suspected that the cancer is localized and confined to the prostate (TNM of T1 and T2), surgical intervention (radical prostatectomy, RP) or radiotherapy (or both) is often used successfully. Although it is more invasive and involves the risk of sexual dysfunction and incontinence, RP is arguably preferable as it is more clinically informative. Following surgery, pathologists re-assess the state of the disease and determine the pathological stage of the RP specimens. The pathological stage is also based on the TNM scale but is more accurate than the clinical stage because of the added information provided by the surgery and the RP specimen. Notably, the presence of cancer at the surgical margins is also assessed because it is often indicative of whether or not the disease is likely to re-occur following surgery [426]. The re-occurrence of PCa is detected through PSA monitoring. When PSA levels are found to reach a certain threshold (usually set between 0.2 to 0.4 ng /ml [430], the disease is considered to have relapsed. This event is termed Biochemical recurrence and occurs in roughly 35% of patients having undergone RP. Of note, patients that relapse within two to three years are at higher risk of dying from recurrent PCa [431].

The use of RP is rarely employed when the clinical TNM stage is of T3 or T4 (extending beyond the prostate) as this procedure does not remove the cancer

that extends beyond the prostate. Since the pioneering studies of Huggins and Hodges in the early 1970s [346], the mainstay for these advanced cases, as well as for recurrent PCa, has been androgen deprivation therapy (ADT) (Sooriakumaran, 2006 #466; Jani, 2006 #470; Sharifi, 2005 #471}. Whereas ADT in combination with radiotherapy is most frequently used for locally advanced cancers [432], ADT is the currently the principal treatment option [433]. ADT is achieved by two principle means. The first is by decreasing blood testosterone levels either by surgical castration (orchiectomy) or through the use of LHRH analogs which cause long term reduction of LH production and inhibition of testosterone secretion by testicular Leydig cells (chemical castration) [283]. The second is through the direct inhibition of the AR using inhibitory androgen analogs such as flutamide and Bicalutamide (Casodex) that compete with DHT for the ABD of the AR [434]. Notably, LHRH analogs and AR inhibitors are often used simultaneously to increase treatment eficacy[435].

1.3.8. Androgen-Independent Prostate Cancer

Although ADT often initially causes massive apoptosis of both normal and cancerous AR-expressing prostate cells, PCa cells almost invariably resist the treatment, leading to what is called androgen-independent PCa (AIPCa) [436, 437]. Presently, there are few available options for patients once AIPCa develops, all of which are palliative in nature. Compounding the problem, studies have suggested that AIPCa cells are also more resistant to other forms of therapy [438, 439]. There are an increasing number of studies that altogether demonstrate the existence of multiple "molecular paths" that can be undertaken by PCa cells in order to acquire the AIPCa phenotype [440]. At the core, these pathways can be essentially divided into two categories: those that involve the AR and those that do not.

1.3.8.1. Androgen Receptor-Dependent Pathways

AR-dependent pathways to AIPCa can be further subdivided into two categories. The first category groups all genetic mutations that can directly affect the expression, structure and function of the AR. This includes mutations within

the ABD such as that observed in the LNCaP AR that can be activated by the anti-androgen flutamide [412]. Other mutations may affect the transactivation domains, such as the amplification of the poly-glutamine tract or the point mutations present in the AR of 22Rv1 cells [409]. The hinge region of the AR is also frequently mutated and though this may yield an AR with delayed nuclear translocation kinetics, some of the mutations also result in heightened transactivation potential [364]. Also falling under this category, AR gene amplification is observed in close to 30% of AIPCa cases [441]. The second category of AR-dependent pathways includes those indirectly affecting AR activity. For example, the up-regulation of several ARA family members has been reported in AIPCa [442]. In fact, over-expression or constitutive activation of several of the AR-interacting co-activators listed in section 1.3.5 has been suggested to promote AIPCa. In addition, hyper-activation of the MAPK pathway has been found to increase AR activity through its phosphorylation [387].

1.3.8.2. Androgen Receptor-Independent Pathways

Fully AR-independent mechanisms of resistance to ADT can operate even in the complete absence of the AR. Prime examples of cells that employ these pathways are the PC3 and DU145 cell lines. However, the prevalence of these pathways in AIPCa patients is somewhat unclear, as most AIPCa specimens express the AR [363, 441, 443, 444]. Nonetheless, deregulations in two potentially closely linked pathways appear to permit AIPCa cell survival, even in the absence of the AR. The first directly leads to the inhibition of apoptosis. Several studies have shown that AIPCa cells can resist ADT through the up-regulation of anti-apoptotic proteins. In particular, the up-regulation of BCL-2-family members BCL-2 [438], BCL-XL [445] and MCL-1 [446] have been found to contribute to AIPCa cell survival. In close association to this, hyper-activation of the PI3K signaling axis is also thought to be of significant importance for AIPCa cell survival, even in AR negative cells [447, 448]. As mentioned previously, hyper-activation of the PI3K/Akt pathway can lead to the inactivation of pro-apoptotic proteins such as BAD, the activation of the mTOR pathway, and activation of NF-κB. As mentioned previously, NF-κB is thought to regulate the

expression of several anti-apoptotic BCL-2-family members and IAPs (see sections 1.2.3.2.5 and 1.2.3.3.3).

1.3.8.3. Current Treatment of Androgen-Independent Prostate Cancer

Currently, the treatment of AIPCa does not go beyond palliation, with the principle outcome of the treatment being reduction of pain and symptoms associated to the development of metastases, particularly those developing in the bones. Today, chemotherapy using the taxane family chemotherapeutic agent docetaxel, which targets microtubules is the standard of care for AIPCa [449]. However, other agents used also include inhibitors of adrenal testosterone production such as ketoconazole as well as injectable strontium or samarium radioisotopes. Bisphosphonates are also increasingly used to control pain due to bone metastases and to reduce complications due to bone fractures, which are typically associated to LHRH analog-induced ADT [450-452]. As LHRH analog therapy also impacts estrogen production, this results in bone demineralization and osteoporosis [453]. However, recent studies suggest that higher doses of bisphosphonates such as Zoledronic acid can also directly prevent PCa cell proliferation and survival [454].

1.4. Emerging Therapeutic Strategies in the Management of Prostate Cancer

In a clinical context, it is of critical importance to maximize the impact of the treatment while minimizing the side effects that can affect patient quality of life. As the aging population is increasingly at risk of developing cancer, the successful application of this concept is likely to have major repercussions on both cancer mortality rates and the overall cost of healthcare.

1.4.1. Chemoprevention

An emerging concept in PCa management is that of chemoprevention [455]. This involves the chronic administration of a non-toxic, minimally insidious compound that prevents or delays cancer progression. Recently completed Phase III clinical trials looking at the potential utility of finasteride, an

inhibitor 5-α-reductase (that converts testosterone to DHT, see section 1.3.2), found that this agent could reduce the occurrence of PCa by 25% [456]. However, finasteride use for chemoprevention is not universally accepted, as some have suggested that it may also increase the incidence of high-grade cancer [457]. However, demonstrating the growing interest for chemoprevention strategies, the large-scale U.S-based SELECT clinical trial assessing the potential of the naturally occurring phytochemicals lycopene, vitamin E and selenium, was initiated in 2001 in response to promising retrospective studies [458, 459]. Although the results are not yet available, a similar NCIC-funded study initiated in 2003 looking at a combination of vitamin E, selenium and multi-component soy extract is also currently being conducted [460].

1.4.2. Molecular markers

In parallel with chemoprevention, a considerable effort has been devoted to isolate molecular markers that can help to predict PCa progression and to predict the response to currently available therapies. Some of these markers were briefly alluded to previously (sections 1.3.3 to 1.3.4.). However despite the many candidates, molecular markers have yet to reach the bedside and join the ranks of currently available clinical and pathological markers (such as those described in section 1.3.7) to help guide clinicians in their choice of treatment.

1.4.2.1 Methods for the evaluation of molecular markers

Molecular markers are typically evaluated retrospectively for their ability to predict an outcome that has in fact already occurred. To this effect selected archival prostate specimens from PCa patients are probed for the presence of molecular markers either by *in situ* hybridization or using immuno-histochemical techniques. Markers are generally quantified semi-subjectively and their prevalence is subsequently confronted to patient outcome. This is very often done following the dichotomization of marker expression according to a threshold value. Although this threshold is sometimes determined empirically, it is frequently obtained optimally through statistical methods such as receiver

operating characteristic (ROC) curves, p-value optimization, or recursive partitioning. Threshold determination by ROC, a statistical method initially developed for assessing diagnostic test efficacy, is based on the sensitivity and specificity of the dichotomization in relation to outcome prediction [461]. The optimal p-value method simply consists of finding the cut-point that yields the lowest p-value with respect to the resulting prognostic groups [462]. Finally, recursive partitioning consists of choosing as the threshold a marker expression value that separates the patient cohort into the two most homogenous subgroups that, between themselves, exhibit the greatest difference with respect to another given characteristic such as the onset of BCR for example [463] (see section 1.3.7.3).

Notably, the recent invention of the tissue micro-array has greatly facilitated the process of molecular marker identification by allowing for the probing of hundreds of small tissue cores in a single experiment. This technological advance has led to the evaluation of an increasing number of candidate markers. This large amount of information has also led the application and elaboration of statistical models, such as multivariate Cox analyses, nomograms and survival trees (that can be obtained by recursive partitioning, see chapters III and IV). Importantly, while all of these statistical models can be useful, each has particularities that render them more or less appealing in certain contexts. Notably, the Cox models are the most frequently used and though these allow the use of both dichotomized (e.g high vs. low) and continuous values, they are difficult to interpret in a practical sense and do not perform well when there are complex interactions between multiple markers. Nomograms are more practical for clinical assessment of patient risk but they do not provide much information pertaining to the relationships between molecular markers. Finally, survival trees present a particularly appealing and easily interpretable graphical format, allowing for the visualization of multi-marker interactions. However, in contrast with the two other methods, the dichotomization of marker values is inherent to the survival tree algorithm [464-468].

1.4.2.2. Prognostic Biomarkers for Predicting Prostate Cancer Relapse

The ideal molecular marker, in the context of the initial PCa diagnosis, is one that accurately predicts whether a patient should undergo surgery, radiotherapy, or none of the above. However, in the post-surgical context, markers that could predict whether or not PCa patients will suffer from recurrent disease following RP would also be very useful. As ADT can have major repercussions on patient quality of life and is currently the primary option for recurrent cancer, patients would likely benefit from more targeted intervention at this crucial stage. This point is also highlighted by recent findings in transgenic mice expressing the Myc oncogene, where it has been found that mice castrated shortly following the apparition of PCa tumor exhibited complete tumor remission whereas residual PCa cells were detected in those castrated later on [341].

1.4.2.2.1. Cell Cycle Markers

Although different studies often disagree on prognostic marker abilities, and although the biological significance of the findings may be counter-intuitive in some cases, several molecular markers have been reported to predict patient prognosis in terms of either biochemical recurrence or patient survival following RP. Interestingly, most of these markers are linked to the cell cycle. For example, in one study, loss or decreased pRb has been found to predict poor patient survival [469] although others have failed to observe this [470]. Similarly decreased $p27^{Kip1}$ expression was found to be associated to poor prognosis [471-474], although in contrast, increased p16(INK4B) [475-477], and increased $p21^{Cip1}$ [478-480] were linked to poor patient outcome. In addition, increased cyclin D and cyclin A [481] were both found predictive of unfavorable prognosis. Genomic amplification of the MYC gene was also concluded to be a poor prognostic indicator in at least two studies [318, 482]. Finally, increased p53 nuclear accumulation has been linked to the onset of biochemical recurrence in several studies [483-492].

1.4.2.2.2. Apoptosis Markers

Many other markers relate to apoptosis. Notably, a high expression of BCL-2 is linked to adverse clinical outcome following RP [489-493]. Perhaps in relation, evidence suggests that nuclear NF-κB p65 is also associated to biochemical recurrence and poor clinical prognosis, including increased risk of pelvic lymph node metastases [494, 495]. In contrast however, at least three studies have found that an increasing number of apoptotic cells (apoptotic index) is adversely prognostic [496-498]. Nonetheless, besides a number of very conflicting studies pertaining to the relationship between BAX and PCa prognosis, few studies have aimed to assess the contribution of pro-apoptotic proteins to PCA prognosis [499-503] (see chapter III).

1.4.2.2.3. The Androgen Receptor

It is particularly interesting to note that even given the ample suggestions pointing to the AR as an important player throughout the development of both PCa and AIPCa, the utility of the AR as a prognostic factor in the specific case of recurrent disease is blurred by contradictory findings [504-508].

1.4.3. Emerging Therapies in Prostate Cancer Treatment

As mentioned previously, therapeutic regimens using docetaxel are the current standard of care for AIPCa patients (see section). However, it is important to note that docetaxel alone or in combination with other available therapies has relatively modest effects on the overall survival of AIPCa patients, generally increasing the median survival by two to three months [509]. As such, there is an increasing effort to expand the arsenal of available therapeutic options.

1.4.3.1. Single-Target Strategies

Several ongoing pre-clinical studies and clinical trials have been initiated using a number of different strategies to address the problem of AIPCa. Many of these aim to inhibit specific targets. For example the BCL-2 antisense G3139 (Oblimersen) has recently completed phase II clinical trials in combination with docetaxel for treatment of AIPCa [510]. Relating to the AR, some pre-clinical

strategies have aimed to use peptide mimeticks to block the interaction between the AR and its co-activators [511]. Others using radioisotopes linked to antibodies (J591) targeting the AR-regulated prostate-specific membrane antigen (PSMA) are being evaluated in combination with chemotherapy [512]. In addition, much attention has been put towards the development of therapeutics targeting the receptor tyrosine kinase pathway. While most target the EGFR, (gefitinib, cetuximab, trastuzumab, lapatinib and erlotinib) [440, 513], others target platelet-derived growth factor receptor (PDGFR) such as imantinib and leflunomide [514, 515] or the VEGF receptor (VEGFR) such as Avastin (bevacizumab) [516]. Other promising single-target strategies involve inhibitors of the mTOR pathway such as CCI-779 and RAD001[517, 518]. Although only at the pre-clinical stage, CCI-779 has shown promising results in a mouse xenograft model in combination with docetaxel [518].

1.4.3.2. Multi-Target Strategies

While many strategies have taken advantage of specific targets and aim to inhibit specific signaling pathways, others strategies aim to affect diverse targets simultaneously through a single agent. In contrast with the truly single target approach, it is thought that this strategy should decrease the potential for recurrent disease because it limits the "available" pathways that may lead to AIPCa cell survival. To this effect, chaperones have been emerging as interesting targets, For example, the Hsp90 inhibitor 17-Allylamino-17-Demethoxygeldanamycin (17-AAG) has recently undergone phase II clinical trials with promising results [519, 520]. Notably, Hsp90 is chaperone to and regulates several key client proteins including the AR (see section 1.3.5.5), Her-2/Neu, Raf, CDK4, Akt, p53, HIF-1α, survivin and telomerase [519, 520]. Similarly, anti-sense therapy against clusterin, a cytoprotective chaperone whose clients are involved in apoptosis and DNA-repair, is currently undergoing phase II clinical trials in combination with chemotherapy [521].

1.4.3.2.1. Proteasome Inhibitors

Another strategy that is likely to affect multiple targets is the inhibition of proteasome degradation. These inhibitors usually consist of small synthetic peptides that compete for the chymotryptic binding site of the proteasome. Many proteasome inhibitors are routinely used for *in vitro* research (e.g MG-132 and ALLN); however, Bortezomib is the main clinical representative of this class of drugs, having been recently approved by both the FDA and the EMEA for recurrent multiple myeloma [522]. Bortezomib has also shown promising results in early phase clinical trials for AIPCa [523, 524], although one recently completed phase II trial suggests it may add little benefit to standard chemotherapy using docetaxel [525]. Notably, proteasome inhibitors may sensitize to various therapeutic insults; however, the mediators of these effects remain controversial. Although the activity of proteasome inhibitors was initially attributed to the inhibition of NF-κB through decreased degradation of IκB proteins, increasing evidence suggests that proteasome inhibitors may affect the expression of various proteins, including cell cycle inhibitors, p53, and pro-apoptotic proteins [522] (dicussed in chapter V).

1.4.4. Inositol Hexakisphosphate

As mentioned previously, one of the potentially active anti-cancer components present in soy is inositol hexakisphosphate (IP6). IP6 was the first inositol phosphate to be discovered [526] and was initially tested for its anti-cancer properties over 15 years ago, following epidemiological evidence suggesting that diets rich in IP6 were inversely linked with colon cancer incidence [527]. Since then, IP6 has been reported to exhibit both chemopreventive and chemotherapeutic activity against several malignancies. *In vitro,* IP6 has been shown to inhibit the proliferation and/or induce the death of erythroleukemia [528, 529], colon [530, 531], lung [532], liver [533], mammary [534], cervix [535], skin, soft tissue and prostate cancer cells [531, 536, 537] (see chapters II and V). Administered mostly in the drinking water or as a dietary supplement, the *in vivo* anti-cancer activity of IP6, has also been reported in various mouse models

of colon [538-547], liver [548, 549], lung [550, 551], breast [543, 547, 552-554], skin [555] , soft tissue [556, 557] and prostate cancer [558]. Importantly, *in vivo* studies suggest that IP6 can be effective as a dietary supplement and that it does not elicit noticeable secondary effects [527].

1.4.4.1. The Synthesis of Inositol Hexakisphosphate

IP6 consists of a 6-carbon ring myo-inositol backbone that is mono-phosporylated at every carbon via hydroxyl groups. Although IP6 is particularly abundant in soy, legumes, and fiber-rich foods, where it is thought to act as a phosphate store, IP6 is present at concentrations of 10-60 μM in almost all mammalian cells [559]. IP6 is produced by the successive phosphorylation of lower inositol phosphates by several inositol kinases. In humans, the enzyme that catalyses the final step in the formation of IP6 has been recently identified as 1,3,4,5,6-pentakisphosphate 2-kinase (Ins5P2K) [560].

1.4.4.2. Inositol Hexakisphosphate and Cell Signaling

Endogenous IP6 and inositol poly-phosphates are thought to play important roles within the cell. For example, signaling via phospholipase C (PLC) leads to the hydrolysis of cell membrane-bound PIP2 (phosphatidyl-inositol-2-phosphate) into diacylglycerol (DAG) and inositol-1,4,5 tri-phosphate (IP3) [212]. As mentioned previously, PIP2 is a substrate of PI3K whose phosphorylation results in the activation of PDKs and downstream Akt (see section 1.2.4.1.2). While DAG plays a role in the activation of PKC, IP3 is involved in the opening of endoplasmic reticulum calcium stores via IP3 receptors (IP3R) [561, 562]. As mentioned above, the successive phosphorylation of IP3 leads to the production of IP6. Several studies point to a role of IP6 in endocytosis/exocytosis and signaling functions at the plasma membrane. In pancreatic β-cells, IP6 was shown to prevent insulin exocytosis by inhibiting the activity of PKC [563]. Again in pancreatic cells, IP6 was found to inhibit the activity of protein phosphatases such PP1, PP2A and PP3, and lead to L-type calcium channel opening [564]. In neural cells, IP6 was found to bind to

synaptotagmins and regulate the secretion of neurosecretory vesicles [565, 566]. In addition, components of the caveolin-1/clathrin-mediated endocytosis pathway such as AP-2 [567] and AP-3/AP180 [568, 569] have also been reported to bind IP6. In this context, IP6 was found to prevent the assembly of clathrin cages required for endocytosis. Still at the level of the plasma membrane, the inhibitory effect of Arrestin on G-protein coupled-receptor activity was reported to require the presence of IP6 [570]. Finally, addition of IP6 at physiological doses (25μM) was recently found to stimulate a 3-fold increase in CKII activity *in vitro* [571]. Interestingly however, except for some indications that IP6 concentrations may change during the cell cycle, cellular concentrations of IP6 are thought to remain stable even following cell stimulation [572].

1.4.4.3. Inositol Hexakisphosphate in DNA repair and mRNA export

Other potentially important functions of endogenous IP6 are linked to the nuclear compartment. IP6 was found to stimulate non-homologous end-joining DNA-repair by binding the Ku70/80 subunits of DNA-PK [573, 574]. Yeast genetic studies established a potential role for IP6 in mRNA export [575, 576]. This was recently confirmed in a study that showed that IP6 stimulates the activity of the Dbp5 RNA-dependent ATPase in collaboration with the Gle-1 protein [577]. As mentioned previously (section 1.1.3) this pathway is particularly important for the export of PolyA-containing mRNA.

1.4.4.5. The Many Potential Anti-Cancer Mechanisms of IP6.

Although it appears that IP6 may function by several mechanisms simultaneously, the specific molecular mediators of the anti-cancer activity of IP6 have not been clearly identified.

1.4.4.5.1. Metal Chelation and Anti-Oxidant Properties

One mechanism that was proposed to mediate the chemopreventive abilities of IP6 activity was through its role as an anti-oxidant. The negatively charged phosphates in position 1,2 and 3 constitute a unique (axial-equatorial-axial) conformation that confer antioxidant properties to IP6 by chelating Fe^{3+} and

preventing Fe^{3+}-catalyzed hydroxl radical formation [578-580]. IP6-mediated chelation of other metals such as Zinc, Calcium and Magnesium has also been proposed to play a role due to the potential role of these divalent cations in cell proliferation [547, 581, 582].

1.4.4.5.2. Effects on Cell Membrane and Signaling

Using supra-physiological doses of IP6, others have found more direct evidence that IP6 may exhibit an anti-tumoral response by modulating cell signaling, particularly at the level of the cytoplasmic membrane. Notably, one study in DU145 cells showed that IP6 inhibits the association between AP-2 and EGFR. The loss of this interaction was paralleled with reduced TGF-α-induced EGFR signaling through the Shc/MAPK pathway. The phenomenon was suggested to be linked to a decrease in AP-2-mediated EGFR endocytosis. This study also showed that IP6 inhibited TGF-α- induced PI3K and Akt activation and suggested that this event results in the inhibition of PI3K/Akt/Rab5- mediated fluid-phase endocytosis, as evidenced by decreased DU145 uptake in HRP-linked albumin [537]. These events were associated to a reduction of DU145 cell proliferation. Inhibition of EGF and TPA-induced PI3K and MAPK activity was also reported in JB-6 murine epidermal cells. This led to the downstream inhibition of AP-1 transcriptional activity and prevented EGF and tetradecanoylphorbol 13-acetate -induced transformation of JB-6 cells [536]. IP6-mediated inhibition of UVB-induced AP-1 activity via ERK1/2 and JNK was also observed in JB-6 cells in another study [583]. On the other hand, two studies did not find that IP6 reduced TNF-α-induced (HeLa cells) or UVB (JB-6 cells) induced PI3K activation [535]. However, both studies found that IP6 inhibited NF-κB activity induced by these agents, and in Hela cells, IP6 also inhibited Akt activation (phosphorylation). In relation to this, constitutive NF-κB activity in DU145 cells was reportedly inhibited by treatment with IP6 [584].

1.4.4.5.3. Effects Relating to the Cell Cycle and Apoptosis

Another possible mechanism mediating the anti-cancer effects of IP6 is through the inhibition of the cell cycle and the induction of apoptosis. One study

demonstrated that treatment with IP6 could block DU145 cells in the G1 phase of the cell cycle. This was associated with increased expression of $p21^{Cip1}$ and $p27^{Kip1}$, decreased *in vitro* CDK6/4-mediated phosphorylation of Rb and reduced *in vitro* CDK2-mediated phosphorylation of histone H1, as well as increased native DU145 pRb phosphorylation leading to a reduced interaction with E2F4. In this study treatment with IP6 also led to the cleavage of PARP and caspase-3 and a modest increase cell-surface presentation of phosphatidyl-serine [585]. Similar findings were observed in LNCaP cells, where decreased AKT activity, increased BAX and decreased BCL-2 expression were also detected [586]. As we will see in chapter V, the activity of IP6 in PC3 cells may be at least partially due to the modulation of the expression of several BCL-2 family members at the protein level. On the other hand, suggesting that IP6 may have cell-type specific effects on the cell cycle, treatment with IP6 leads to cell cycle arrest at the G2/M phase in some leukemia cell lines [528] instead of in G1 phase as observed in PCa cell lines. Of note, this study also found that IP6 had no effect on normal bone marrow specimens.

1.4.4.5.4. *In Vivo* Effects

At least *in vivo*, the effect of IP6 may be linked to other effects such as vascularization, immune response and tumor invasion. In what constitutes the only *in vivo* study looking at the effect of IP6 on PCa progression, DU145 xenograft tumors obtained from mice given 2% IP6 in drinking water exhibited decreased microvasculature and VEGF expression, as well as increased expression of IGF-binding protein-3 (IGFBP3), a secreted protein that binds and antagonizes IGF growth factors [558]. In another study, IP6 was found to antagonize bFGF binding [587]. IP6 was also found to enhance natural killer (NK) immune cell activity *in vitro* and prevented carcinogen-induced repression of NK cell activity *in vivo* [588]. Finally, IP6 was reported to inhibit MMP-9 secretion in MDA-MB 231 breast cancer cells, suggesting it may halt tumor infiltration and metastasis [589].

2. Doctoral thesis objectives

The over-arching goal of this doctoral project was to innovate in the treatment of PCa and to further our understanding of the factors involved in both clinical outcome and therapeutic response. To this effect, we initially turned our attention to IP6. The evidence presented in section 1.4.4. initially suggested that treating cells with IP6 could potentially affect several molecular pathways simultaneously. Analogously to the strategies employed by emerging anti-cancer agents such as 17-AAG and proteasome inhibitors, we hypothesized that a multi-targeted therapeutic approach could be beneficial for the treatment of AIPCa, particularly given the multiple pathways that lead to the AIPCa phenotype. Because the effects of IP6 in the context of PCa had been thus far studied almost exclusively in DU145 cells, one of our first goals was to establish whether IP6 could be effective in other PCa cell lines.

This was addressed in chapter II. The results of the study confirmed that IP6 was active in at least three other PCa cell lines, although its effect was evidently greater in AR-negative AIPCa cells. In light of this, we proceeded to determine whether the AR could protect cells from the effects of IP6, using the PC3/PC3(AR) cell lines as models. We found that at least in PC3 cells, an androgen-independent AR-linked function could decrease the efficacy of IP6. Because of other studies suggesting that AIPCa is frequently also resistant to chemotherapy [438, 439], we set out to understand how the AR could mediate this effect. Evidence from the literature known to us at that time suggested that IP6-treated DU145 cells exhibited impaired PI3K/Akt/NF-κB signaling and underwent Rb/E2F-mediated G1 cell cycle arrest and apoptosis (see section 1.4.4.2.2 and 1.4.4.2.3). So-guided, we looked at the effect of the AR on IP6-induced changes in the mRNA expression profile of a subset of relevant genes. We found that in parallel with reducing the manifestations of apoptosis, expression of the AR in PC3 cells prevented the IP6-induced up-regulation of puma and noxa, as well as of the NF-κB-responsive genes IκB-α and irf-2 at the mRNA level.

Following this initial study, it became apparent that the pro-apoptotic NOXA and PUMA could be potential downstream effectors of IP6 action (a possibility we further explored in chapter V). As such, it seemed relevant to determine whether these proteins were expressed in PCa patients. Importantly, the expression of NOXA and PUMA had not yet been characterized in prostate tissues. As mentioned in section 1.4.2.2.2, very little is known concerning the contribution of pro-apoptotic proteins to PCa clinical outcome. Consequently, we were also interested in determining whether NOXA and PUMA expression could be useful markers for PCa prognostic, as this could impact the use of currently available treatments and foreshadow the potential usefulness of IP6 for PCa treatment. These questions were addressed in chapter III.

Beyond identifying NOXA and PUMA as potential mediators of the effect of IP6, the results obtained in chapter II suggested that an androgen-independent AR function could negatively impact IP6 efficacy. Therefore, we set out to assess the expression of the AR in prostate tissues and to determine how AR expression related to the risk of recurrent disease. Although others have previously evaluated the prognostic ability of the AR regarding BCR following RP, there is to date no consensus on its prognostic utility (see section 1.4.2.2.3). Based on evidence from the literature (see section 1.3.5.6) and results from chapter II suggesting potentially important cytoplasmic roles of the AR, we also deemed it justified to study AR sub-cellular localization. Hence, we set out to study the expression and sub-cellular localization of the AR in prostate tissues and to evaluate its ability to predict recurrent PCa in chapter IV.

Because the precise target(s) of IP6 remain somewhat unclear, we continued our exploration of the mechanism of IP6 in the context of PC3 cells. Flowing from the observations made in chapter II, we proposed that the up-regulation of pro-apoptotic genes such as noxa, puma and bax could be involved in mediating the effects of IP6. We also suggested that, in contrast with other reports, the NF-κB pathway could be triggered by treatment with IP6. These possibilities were explored in chapter V. An underlying long-term goal of this endeavor was to further understand the influence of the AR on the effects of IP6.

As opposed to a more direct AR-focused strategy, we opted for this rather indirect route in light of the multiple binding partners of the AR (as presented in section 1.3.5) and of the hundreds of AR-regulated genes. Guided by the over-arching goal of innovating in the treatment of PCa, we also reasoned that better understanding the mechanisms of IP6 could lead to the improvement of its therapeutic impact for use either alone or in combination with current and emerging PCa treatment modalities.

CHAPTER II

An Androgen-Independent Androgen Receptor Function Protects from Inositol Hexakisphosphate Toxicity in the PC3/PC3(AR) Prostate Cancer Cell Lines

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Author contributions

For this paper, I did the majority of the experiments and wrote the paper in its entirety although all co-authors played a role in the editing process. Benjamin Péant and Laurent Lessard helped with western blotting, whereas Nathalie Delvoye provided assistance with cell culture as well as RNA extractions.

Keywords: Androgen Receptor, Inositol Hexakisphosphate, Androgen-Independent Prostate Cancer, E2F, NF-κB.

Abstract

BACKGROUND: Inositol hexakisphosphate (IP6) is a phytochemical exhibiting anti-cancer activity. Because few prostate cancer (PCa) cell lines have been used to study IP6, we assessed its efficacy in a panel of PCa cell lines. METHODS AND RESULTS: Using WST-1 assays we observed that, although androgens did not modulate its efficacy, IP6 was more active in androgen receptor (AR) negative cells than in AR-positive cells. Stable expression of the AR in PC3 cells (PC3(AR)) decreased the response to IP6, which was reversed by an AR-targeting siRNA. Furthermore, AR expression in PC3 cells resulted in significantly reduced caspase-3 activation ($p<0.001$) and DNA fragmentation ($p<0.05$) in response to IP6. Similarly, although treatment with IP6 caused the up-regulation of NF-κB-responsive (IκB-α, IRF-2) and p53/E2F-responsive genes (Puma, Noxa) in PC3 cells, this increase was reduced in PC3AR cells ($p<0.01$). CONCLUSION: We conclude that resistance to IP6 can be linked to a ligand-independent AR function.

Introduction

One of the major issues in prostate cancer (PCa) treatment is the appearance of androgen-independent (AI) PCa that progresses in the face of androgen deprivation therapy (ADT) and is generally resistant to conventional therapeutic modalities. This advanced stage of the disease, which develops in roughly a quarter of PCa patients, is associated with a very poor clinical outcome resulting from its highly aggressive metastatic phenotype and its resistance to chemotherapy (1, 2).

The target of hormone-therapy is the androgen receptor (AR), a nuclear receptor family transcription factor important in prostate cell survival (3). The AR is composed of two trans-activation domains and a DNA-binding domain that recognizes androgen response elements. While the C-terminal trans-activation domain of the AR requires androgen binding to activate gene transcription, the N-terminal trans-activation domain does not (4). In addition to its known role as a transcription factor, evidence suggests that the AR may also hold other functions although these are not well understood. For example, it has been shown that Rb interacts with the AR in an androgen-independent fashion (5). In addition, the AR forms complexes with cyclin D1(6) and interacts directly with cyclin E (7). The AR has also been found to exhibit mutual transcriptional inhibition with the NF-κB subunit RelA (8) and has been reported to interact with and act as a substrate for Akt kinase (9).

Aberrant AR activation or expression is frequently observed in AIPCa and is believed to at least partially mediate its resistance to ADT (10, 11). In addition, although it is not clear how these become prominent in response to androgen ablation, multiple pro-survival signaling pathways have been found to be hyper-activated in AIPCa cells. For example, up-regulation of anti-apoptotic proteins such as BCL-2 (12) as well as deregulated activation of mitogen activated protein kinases (MAPK/ERK) (13), protein kinase C (PKC) (14), jun kinase (JNK) (15), AP-1 (16), ß-catenin (17), E2F (18), ErbB family growth factor receptors and NF-κB (19-22) have been observed in various PCa models. This has led some to suggest that targeting multiple pathways simultaneously could be a good strategy

to effectively manage AIPCa (23).

With respect to this idea, phytochemicals are a rich source of candidate multi-target compounds, having the added advantage of being generally well tolerated by patients (reviewed in (24)). One of these multi-target phytochemicals is phytic acid or Inositol hexakisphosphate (IP6). IP6 is found most abundantly in soy and legumes and has demonstrated anti-cancer activity in a wide range of cancers (25). Although definite mechanisms have yet to be established, IP6 activity has been reported to involve several processes (26). In relation to PCa, IP6 was found to inhibit constitutive NF-κB activity (27) and induce apoptosis of DU145 cells by inhibition of fluid endocytosis and ErbB1 receptor signaling through the MAPK signaling cascade (28). It was also reported that IP6 modulates cdk-cyclin and pRb/E2F complexes, up-regulates p21 and p27 cell cycle inhibitors, causing DU145 cells to block in G1 and undergo apoptosis (29). Because most studies pertaining to IP6 in PCa have focused on the AR-negative DU145 cell line, we set out to compare the *in vitro* efficacy of IP6 on both androgen-sensitive and androgen-insensitive PCa cell lines.

Materials and Methods

Cell culture: 22Rv1, PC3, LNCaP and DU145 were obtained from ATCC (Rockville, MD). The PC3(AR) that stably over-expresses full length human AR (denoted as $PC3(AR)_2$ in Heisler et al. 1997) was a kind gift from Dr. T.J. Brown (University of Toronto) and was also derived from PC3 cells provided by ATCC. Except for 22Rv1 cells, which were maintained in DMEM, cells were maintained in RPMI 1640 complemented with 10% FCS (or charcoal-stripped FCS), gentamicin (50 μg/ml) and amphotericin B (250 ng/ml) (Gibco-BRL, MD, USA). The PC3(AR) cell line was maintained in charcoal-stripped media and complemented with 100 μg /ml Hygromycin B. Cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere and sub-cultured at 1:3 (LNCaP), or 1:5 (PC3, PC3AR, 22Rv1, DU145) by trypsinization with 0.25% trypsin for 5-10 minutes at 37°C (Gibco-BRL, MD, USA).

WST-1 metabolic assay: Cells were grown to confluence, trypsinized, and counted on a hemocytometer. For experiments assessing the effect of androgens on IP6 sensitivity, cells were maintained in either charcoal-stripped (steroid-free) 10% FCS-supplemented RPMI 1640, normal 10% FCS-supplemented RPMI 1640, or normal 10% FCS-supplemented RPMI 1640 + 10 nM R1881 (a synthetic androgen analog) for 72 hours prior to seeding. Cells were diluted in the indicated media at 20 000 cells/well (100 µl). Cells were allowed to adhere overnight prior to treatment and were treated with indicated concentrations of myo-inositol hexakisphosphate dodecasodium salt (Sigma-Aldrich, USA) dissolved in water. Another well containing only media was included for WST-1 assay background correction. After 24 hours, 10 µl WST-1 reagent (Roche, USA) was added to the wells and plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. Incubation times were optimized for each cell line as recommended by manufacturer. WST-1 signal was measured on a Bio-Rad Model 3550 microplate reader at 450 nm with reference at 655 nm wavelength. Following data acquisition, cell metabolic activity was calculated by first subtracting the readout of the WST-1 background (media + WST-1) from all values. Subsequently, relative metabolic activity was calculated as being the WST-1 readout of treated well / WST-1 readout of mock-treated well. Average and standard error of the mean were calculated from normalized replicate values as indicated in the figures and cellular metabolic activity was plotted as a function of IP6 concentration.

Determination of cell doubling time: Cells were plated at a density of 100 000 cells per 2 ml serum supplemented media in 6-well plates. 24, 48 and 72 hours following plating, cells were washed with PBS and incubated in 1 ml trypsin at 37°C for 15 minutes. Cells were then counted on a hemocytometer. Cell doubling time was determined using the formula (doubling time= 2/slope) where slope was determined by plotting the fold change in cell number against time. Experiments were repeated twice in triplicate.

Transfection of siRNA: siRNA targeting the AR and the RISC-free siGLO fluorescent siRNA control were purchased from Dharmacon (Chicago, IL, USA). Briefly, cells were seeded at 400 000 cells per 100 mm petri an allowed to grow for 72 hours prior to transfection which was done as recommended by the manufacturer using the Dharmafect 2 transfection reagent. Cells were incubated for 24 hours after which transfection media was replaced with normal 10% FCS-supplemented RPMI 1640. The following day, cells were seeded as described for the WST-1 assay or at 400 000 cells / 2 ml in 6-well plates for protein or RNA extraction

Protein extraction: After treatments, media was aspirated, cells were scraped, washed twice with cold PBS and pellets were frozen at –80°C. Subsequently, whole cell extractions were performed by applying cold lysis buffer (10 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 0.5% NP-40, 0.5 mM PMSF, 0.2 mM orthovanadate, 2 μg/ml of aprotinin, leupeptin and pepstatin) on ice for 30 min. Whole cell extracts were collected after centrifugation in a Heraeus Biofuge (13,000 rpm for 10 min at 4°C) and were immediately stored at -80°C. Protein concentration was measured by Bradford assays (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions.

Western blot analysis: For Western blot analysis, 20 μg of whole cell protein extract were resolved on a 10% polyacrylamide gel and then transferred onto nitrocellulose membranes. Blots were blocked using 5% non-fat dry milk in TBS-Tween 0,05% buffer overnight at 4°C and probed using anti-AR antibody (NeoMarkers, Clone AR-441, MS-443-P) in blocking buffer (1:500) for 1 hour at room temperature. Membranes were then incubated with secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences Inc., Arlington Heights, IL) in blocking buffer for 1 hour at room temperature and developed with enhanced chemiluminescence (ECL) substrate (Amersham Life Sciences Inc., Arlington Heights, IL).

Caspase-3 fluorigenic assays: PC3 and PC3AR cells were plated at a density of 200 000 cells/ml and treated with 2 mM IP6 or water for 24 or 48 hours. Cells and media were collected by scraping and washed twice with PBS. Cells were resuspended in 50 µl lysis buffer (10 mM Tris HCl pH 7.5, 10 mM NaH_2PO_4, 10mM Na_2HPO_4, 13 mM NaCL, 10 mM $Na_4P_2O_7$, 1% Triton X-100) and incubated for 10 min on ice. Supernatants were cleared by centrifugation and dosed using the Bradford reagent (see protein extraction). 70 µg of protein was used per caspase assay. Each sample was supplemented with lysis buffer to reach a final volume of 56 µl to which 44 µl of caspase assay mix was added [32 µl of caspase assay buffer (312.5 mM HEPES pH 7.6, Sucrose 31.25%, CHAPS 0.3125%), 1 µl DTT (1M) and 2 µl Ac-DEVD-AFC caspase-3 substrate (1.82 µg/µl, Biomol International, PA, USA) and 9 µl of lysis buffer]. Fluorescence was measured every minute for 30 minutes at 37°C in a black 96-well plate using a Fluostar Optima (BMG, Durham, NC, USA). Fluorescence was plotted as a function of time and the slope was calculated in Excel using the linear part of the curve. Data is expressed as a treated/untreated slope ratio. Experiments were repeated 2-3 times and caspase activity measurements were done in duplicate for each experiment.

Filter elution assays (DNA fragmentation): Prior to the assay, cells were plated at 400 000 cells in normal 10% FCS-supplemented RPMI 1640 per 100 mm petri (10 ml volume) and labeled with 1 µCi [^{14}C]-thymidine for 72 hours. After labeling, cells were counted and plated at 400 000 cells per 2 ml in 6-well plates and allowed to adhere overnight. The following day, cells were treated with water or 2 mM IP6. Filter elution assays were performed at 24 and 48 hours as described previously (30). Briefly cells were scraped using a rubber scraper and 2 ml of labeled cells were loaded on protein adsorbing 25 mm diameter Metricel membrane vinyl/acrylic copolymer filters, (Pall Corporation, Ann Arbor, MI, USA) and washed with 3 ml PBS. After elution by gravity, lysis was performed with 5 ml of lysis solution (2% SDS, 0.04 M EDTA, pH 10.0). After the lysis

solution had dripped through, the filters were washed with 5mL 0.02 M EDTA (pH 10.0). Filters were then removed and treated with 1N HCl for 1 hour at 60°C to depurinate the DNA. 3 ml NAOH was then added to release labeled DNA from the filters. Radioactivity was counted in each fraction (loading wash, lysis, EDTA wash, filter) by liquid scintillation spectrometry. DNA fragmentation was determined as the DNA fraction in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (sum of the four fractions). The results were expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in untreated cells (background) according to the formula (F-F0/1-F0) X 100 where F and F0 represent DNA fragmentation in treated and control cells, respectively.

Quantitative Real time PCR: PC3 and PC3AR cells were plated at a density of 200 000 cells/ml and treated with 1 mM IP6 or water. After 24 hours, media was removed and RNA was extracted with Trizol reagent according to manufacturers instructions (Invitrogen, ON, Canada). Concentration of RNA samples was determined using a Beckman DU-600 spectrophotometer. 2μg RNA was used to synthesize a cDNA using the SuperScript first-strand synthesis system (random hexamer method) according to manufacturers instructions (Invitrogen, ON, Canada). The QuantiTect SYBR Green PCR kit was used as recommended (Quiagen, ON, Canada). Real time PCR reactions were performed on a Rotor-gene RG-300 (Corbett Research, Australia). Optimal threshold and reaction efficiency were determined using the Rotor-gene software. Melt curves for each primer exhibited a single peak, indicating specific amplification, which was also confirmed by agarose gel. Ct values were determined using the Rotor-gene software at the optimal threshold previously determined for each primer. Relative mRNA/actinB ratios were calculated using the method described by Pfaffl et al. (2001) (31). Fold induction was calculated relative to the mock treated control for each gene. Experiment was done twice and real time measurements were done in duplicate for each gene in each experiment. Primer sequences used were as follows (5' to 3', F= forward, B=backward). Bax

F=AACTGGTGCTCAAGGCCCTG B=GGGTGAGGAGGCTTGAGGAG. Noxa F=TTCGTGTTCAGCTCGCGTCC B=CTCGGTTGAGCGTTCTTGCG. Puma F=TGGACTCAGCATCGGAAGGT B=GCACCAGCACAACAGCCTTT. BCL-2 F=CCGCTACCGCCGCGACTTC B=AAACAGAGGCCGCATGCTG. IκB-α F=CTGGCTTTCCTCAACTTCCA B=GTCTCGGAGCTCAGGATCAC. $BCLX_L$ F=GGTATTGGTGAGTCGGATCG B=TGCTGCATTGTTCCCATAGA. cyclin D1 F=CGTGGCCTCTAAGATGAAGG B= CCACTTGAGCTTGTTCACCA. ActinB F=ACTCTTCCAGCCTTCCTTCC B=GTACTTGCGCTCAGGAGGAG. IRF-2 F=CCATGAATTCCTTGCCTGAT B= AAGCCCCAGAGATGACTCAA. Cyclophilin B F= GCCCAAAGTCACCGTCAAGGTGT B=GCTCTTTCCTCCTGTGCCATCTC. Primers for each target gene were designed with the help of the Primer 3 software (32) with the exception of those for puma noxa and bax that were obtained from Dr. Richard Bertrand and for BCL-2 which were described elsewhere (33).

Statistics: Statistical significance of observed differences between PC3 and PC3AR cells or between siRNA-transfected cells was assessed using a two-tailed, two-sample, unequal variance T-test in Excel (Microsoft, USA). In addition, one-way ANOVA analyses using SPSS v.11 (SPSS Inc. Chicago, USA) were performed in order to confirm the significance of differences in IP6 sensitivity following siRNA transfection obtained by T-Test, as well as to evaluate the significance of differences in cell doubling times. P values below 0.05 were considered as significant.

Results

Prostate cancer cells that do not express the AR are more sensitive to the effects of IP6 on cellular metabolism

To compare the effect of IP6 on PCa cell lines, we used the WST-1 colorimetric assay, which is widely employed for the assessment of compound cytotoxicity (34-37). The activity of cellular and mitochondrial membrane

electron transport enzymes on WST-1 yields a soluble formazan dye that can be measured by spectrophotometry (37). Hence, WST-1 readout correlates to the metabolic activity of the cell. We chose LNCaP, 22Rv1, PC3 and DU145 cell lines to assess the relative efficacy of IP6 so as to include both hormone-sensitive and hormone-insensitive PCa cell lines. Cells were treated with IP6 from 0-3 mM concentrations and cell survival was calculated relative to mock treated control as described in Materials and Methods. Figure 1a) shows that IP6 is strikingly more effective at inhibiting metabolic activity in PC3 and DU145 when compared to LNCaP and 22RV1 cell lines. As shown in figure 1b, no significant differences in cell doubling times were observed between the cell lines ($p>0.05$, ANOVA) Hence, the extent of metabolic toxicity did not correlate with cell growth rate. Similar results were obtained in relation to basal metabolic activity (data not shown).

Androgens do not affect metabolic sensitivity to IP6

One important parameter that differentiates the LNCaP, 22Rv1 and PC3, DU145 cell lines is that the former express the AR whereas the latter do not (reviewed in (38, 39)). In light of this, we determined whether the androgen-dependent activity of the AR could affect the sensitivity of cell lines to IP6. To address this question, we performed WST-1 assays in conditions of androgen deprivation and supplementation. Cells were pre-treated in steroid-free media or media supplemented with 10 nM R1881, a synthetic androgen analog, for 72 hours prior to seeding. Figure 2a-d) show that androgen supplementation does not decrease IP6 sensitivity in AR positive LNCaP and 22Rv1 cell lines. Similarly, androgen deprivation does not sensitize these cells to the effects of IP6 on metabolic activity.

Expression of the AR protects PC3 cells from the effect of IP6 on cell metabolic activity

Because the AR possesses an androgen-independent trans-activation domain (4) and has been reported to interact with certain proteins such as Rb in an

androgen-independent fashion (5), we addressed the possibility that the physical presence of the AR could alter the efficacy of IP6. To this effect, we compared the activity of IP6 on wild-type PC3 cells and a PC3 cell line in which the human AR had been previously transfected (PC3(AR)) and is stably expressed ((40) and Fig. 4a). WST-1 assays performed in normal serum-supplemented conditions showed that although PC3 and PC3(AR) cells divided at similar rates (figure 1b), PC3(AR) cells were significantly more resistant to IP6 than PC3 cells at all concentrations ($p<0.05$) (Fig. 3a). Supporting our previous observations in LNCaP and 22Rv1 cells, androgen supplementation or deprivation did not respectively protect or sensitize PC3(AR) cells to the effects of IP6 on cell metabolic activity (Fig 3b). To further address the role of androgens in this phenomenon, we used the non-steroidal AR inhibitor Casodex, which also had no effect on IP6 efficacy (data not shown).

An AR-targeting siRNA sensitizes PC3(AR) cells to the effects of IP6 on cell metabolism

In order to eliminate the possibility that our observations were due to clonal effects in PC3(AR) cells, we used a siRNA to knock down expression of the AR protein. Figure 3c shows that the siRNA had effectively decreased AR protein expression when IP6 cytotoxicity was assessed. Figure 3d shows that PC3(AR) cells transfected with a siRNA targeting the AR significantly increased IP6-induced metabolic inhibition compared to both mock transfected ($p<0.003$) and siGLO RISC-free siRNA control-transfected cells ($p<0.00001$). On the other hand, siRNA transfection had no effect on PC3 sensitivity to IP6 (Fig. 3e). In contrast to what was observed in PC3(AR) cells, although AR-targeting siRNA effectively reduced AR expression in both LNCaP and 22Rv1 cells (4a), transient knockdown of AR expression did not further sensitize LNCaP or 22Rv1 cells to the effects of IP6 on cellular metabolism (4b-c).

Expression of the AR protects PC3 cells from IP6-induced caspase-3 activation

Although the WST-1 assay is a valid method for determining cellular metabolic activity, it does not necessarily denote cell death per se. It has been previously suggested that IP6 induces caspase-3 activation and apoptosis in other cancer cell lines (26, 29, 41). Hence, we compared caspase-3 activation following a 24 or 48-hour treatment with 2mM IP6. In order to quantify caspase-3 activity, we used the DEVD-AFC fluorigenic substrate that emits light upon cleavage of the DEVD sequence by active caspase-3 or caspase-3-like cysteine proteases (42, 43). Figure 3c shows that increased caspase-3-like activity was detectable in PC3 cells as early as 24 hours following treatment with IP6 and was sustained over 48 hours. Although caspase-3-like activity was also increased by IP6 treatment in PC3(AR) cells, this increase was of significantly lower magnitude both at 24 and 48 hours ($p<0.05$).

Expression of the AR protects PC3 cells from IP6-induced DNA fragmentation

We also looked at DNA fragmentation, a marker of late stage apoptosis, in response to treatment with IP6. While this is typically assessed subjectively by observing DNA laddering on agarose gel, DNA fragmentation can be quantitatively measured using DNA filter elution assays (30). Using this method we followed DNA fragmentation at 24 and 48 hours after treatment with 2mM IP6. We can see from the data presented in figure 3d that although a 14.7% increase in DNA fragmentation was already observable at 24 hours in PC3 cells, an increase of less than 7% was observed in PC3(AR) cells even at 48 hours whereas PC3 cells had reached a 32.8% increase in fragmented DNA at this time. The observed differences between PC3 and PC3(AR) cells pertaining to DNA fragmentation induced by treatment with IP6 were statistically significant at both 24 ($p<0.01$) and 48 hours ($p<0.05$).

IP6 treatment induces the expression of a subset of NF-κB-responsive genes as well as of pro-apoptotic genes in PC3 but not PC3(AR) cells.

Following from the observation that the AR protects PC3 cells from IP6-induced apoptosis, we determined whether this could translate in differences in the induction of gene expression following a treatment with IP6. Using real time PCR, we measured the expression of the pro-apoptotic genes bax, puma and noxa, and of the anti-apoptotic genes BCL-2 and BCL-X_L. Because of reports suggesting that IP6 inhibits constitutive NF-κB activation in DU145 cells (27) as well as TNF-induced NF-κB activation in HeLA cells (41), we also probed for the expression of IκB-α, IRF-2 and cyclin D1, which have been shown to be NF-κB-responsive (44-46). PC3 and PC3(AR) cells were treated with 1mM IP6 and the fold change in gene expression (relative to actin B) was calculated and are represented in figures 4a-h. We observed moderate to strong induction of bax (4.6-fold), puma (6.3-fold), noxa (1.6-fold), IκB-α (18.7-fold) and IRF-2 (3.6-fold) in PC3 cells. In comparison, the fold change in puma (1.3-fold), noxa (0.6-fold), IκB-α (0.8-fold) and IRF-2 (0.9-fold) was significantly reduced in PC3(AR) cells ($p<0.05$ for each gene). Though on average bax induction was also decreased in PC3(AR) (1.1 compared to 4.6 in PC3 cells), this was not statistically significant ($p=0.13$). Minimal changes in the expression of BCL-X_L, BCL-2 and cyclin D1 were observed (between 0.75 and 1.4 fold) although to similar extents in both cell lines. Similar results were obtained using cyclophilin B as a reference gene (data not shown)

Discussion

The evidence presented in figure 1a suggests that there is a link between AR expression and IP6 efficacy as cell lines that express the AR are less sensitive to the metabolic effects of IP6 even though growth rates are similar between all cell lines under the conditions tested here (1b). In contrast, treatment with an androgen analog does not hinder on the ability of IP6 to inhibit PCa cell metabolic activity, nor does androgen deprivation protect from its effect (fig. 2a-d, 3b), suggesting that androgens are not involved in this phenomenon. Surprisingly, steroid-free conditions conferred a slight protection to 22Rv1 cells (fig 2b). This may be due to the lack of other steroids in the culture media resulting from the

charcoal-stripping procedure (47). Regardless, addition of the R1881 androgen analog clearly had little effect on IP6 activity in this cell line.

Further supporting a link between AR expression and IP6 efficacy, we found that stable expression of the AR in the PC3 cell line interferes with the effects of IP6 on cell metabolism and cell death (fig. 3a, 3c-g). However, as opposed to its clear effect on PC3(AR) cell metabolic sensitivity to IP6, short term knockdown of the AR did not modulate the efficacy of IP6 in LNCaP or 22Rv1 cells. These results indicate that while AR may be an important component of response, other pathways may also act to modulate IP6 efficacy in LNCaP and 22Rv1 cells. Nonetheless, these results also demonstrate that the exogenous expression of the AR in the PC3 cell line is a suitable model for studying the AR-associated function involved the resistance to IP6 observed here. In combination with the observation that androgen supplementation or deprivation does not protect or sensitize any of the androgen-responsive cell lines to the effects of IP6 (fig. 2a-b, 3a), these data suggest that a ligand-independent function of the AR hinders on the toxicity of IP6 at least when expressed in PC3 cells.

The results reported here support the notion that the AR may contribute to chemotherapy resistance in AIPCa. Two studies have previously reported on the effect of ligand-mediated AR functions on apoptosis. It has recently been shown that androgens could up-regulate caspase-2 expression and protect PCa cells from TNF receptor family ligand-induced apoptosis (48). In contrast, a separate study found that an androgen-dependent AR activity was required to sensitize PCa cells to paclitaxel and γ-radiation (49). To our knowledge, this is the first report of an androgen-independent effect of the AR on apoptosis induced by a cytotoxic agent.

As a first endpoint to assess the effectiveness of IP6 on PCa cell lines, we looked at cellular metabolic activity using the WST-1 assay. Given that WST-1 is an indicator of metabolic activity and not cell death, and since IP6 had been previously shown to induce apoptosis in other PCa cell lines, we also quantified caspase-3-like activity (fig. 3c) and DNA fragmentation (fig. 3f) as alternate endpoints for comparison. Although it had been previously observed that IP6 inhibits PC3 cell growth and induces their differentiation (50), the observed

increase in DNA fragmentation and caspase-3-like activity supports that IP6 also promotes apoptosis in PC3 cells as has been observed in other cell lines (29, 41). Overall, the differences observed between PC3 and PC3(AR) cells in relation to apoptosis markers correlated with those detected using metabolic toxicity assays. On the other hand, the extent of metabolic toxicity conferred by 2mM IP6 treatment at 24 hours in both PC3 and PC3AR cells appeared to be greater than the extent of caspase-3-like activity and DNA fragmentation at the same time and IP6 concentration (compare fig. 3a to fig. 3c and 3d). This may indicate that the metabolic effects of IP6 occur first. Alternately, this could also suggest that a portion of cells die early on by necrosis.

Real time PCR analyses showed that a subset of NF-κB-responsive genes is up-regulated upon treatment with IP6 in PC3 cells (fig 4f-h). This somewhat contradicts one study where constitutive NF-κB binding to κB sequences in DU145 cells was inhibited by a 24-hour treatment with IP6 (27) and another study in HeLa cells where TNF/insulin-induced NF-κB-luciferase reporter activation was also inhibited by pre-treatment with IP6 (41). Because IκB-α protein is in turn an inhibitor of the classical NF-κB subunits (p50/RelA), it may be that IκB-α up-regulation eventually translates into the net inhibition of NF-κB activity (44). However, the up-regulation of IκB-α and IRF-2, and to a lesser extent cyclin D1, suggests that NF-κB is at least activated within the first 24 hours following treatment with IP6.

Although IκB-α is strongly induced in PC3 cells (18.7 fold, fig 4f), cyclin D1 is only minimally up-regulated (1.4-fold, fig. 4h) and BCL-2 expression is slightly decreased (0.75-fold, fig. 4d) in response to IP6. Interestingly, cyclin D1 and BCL-2 have been shown to be responsive to non-classical NF-κB p52 homo-dimers in breast epithelial cells (45, 51). Conversely, IκB-α is regulated by classical p50/RelA heterodimers (44). If this regulatory network were maintained in PC3 cells, it would be tempting to speculate that the classical as opposed to the non-classical NF-κB pathway is preferentially activated in response to IP6 in this system.

We also observed that the pro-apoptotic protein-encoding genes bax, noxa and puma, but not the anti-apoptotic protein-encoding genes BCL-2 and BCL-X_L were up-regulated in PC3 cells following treatment with IP6. Though characterization of the protein expression and sub-cellular localization of the gene products will be necessary to address whether bax, puma and noxa may be directly involved in IP6-induced apoptosis, it appears to be a likely possibility. Notably, both noxa and puma are p53/E2F1 responsive genes. Given that PC3 cells are null for p53 (38), this supports the notion that IP6 may exert some of its effects through E2F family transcription factors; however, our observations suggest activation rather than suppression of net E2F transcriptional activity as proposed by others (29). This contradiction may be due to the fact that the DU145 cells used in the aforementioned study harbor a mutant pRb, which is an important modulator of E2F function. In addition, the authors observed that although E2F4/Rb complexes increased in reaction to IP6, overall E2F4 was in turn down-regulated. Because the status of the transcriptional activator E2F1 and overall E2F-dependent transcriptional regulation was not assessed, it remains possible that this could result in a net increase of E2F-regulated gene expression, particularly since E2F4 is thought to be repressive in nature (52).

The mechanism underlying the inhibition of IP6-induced PC3 cell death by an androgen-independent AR-associated function remains unclear. The finding that NF-κB and E2F responsive genes are up-regulated significantly less in PC3(AR) cells in response to IP6, compared with PC3 cells brings forth the possibility that the AR may interfere somewhere along one or both of these signaling pathways. Although NF-κB has been shown to promote apoptosis in certain contexts (53), it is generally considered to be anti-apoptotic (54). In the latter case, its initial activation may be a counter-response to pro-apoptotic signals such as those responsible for the up-regulation of Puma and Noxa in PC3 but not PC3(AR) cells. Hence, the E2F pathway may dominate the response to IP6, and may itself be influenced directly or indirectly by the AR. Interestingly, cyclin D1, cyclin E, as well as Rb are known components of the E2F pathway and have all been shown to interact with the AR (5-7). In fact, the interaction between Rb and

the AR was found to be androgen-independent (5). Alternately, since the AR possesses a ligand-independent trans-activation domain, it is also possible that a ligand-independent transcriptional activity of the AR mediates the expression of a currently unidentified protein that modulates IP6 toxicity. Studies addressing these possibilities are currently underway.

Conclusions:

We conclude that there is a link between AR-expression and sensitivity of PCa cell lines to IP6. Furthermore, we show that this could involve a ligand-independent AR function that can protect PC3 cells from the effects of IP6 on cell metabolism and subsequent apoptosis. We also conclude that IP6 induces the expression of NF-κB-responsive genes as well as E2F-responsive genes in PC3 cells that may be involved in IP6-induced apoptosis. In addition, we find that stable expression of the AR in PC3 cells significantly diminishes NF-κB and E2F-responsive gene up-regulation. Overall, this suggests that, in addition to its role in ADT resistance, the AR could contribute to AIPCa resistance to chemotherapy as well. We believe that IP6 will be a useful tool to explore this newly discovered property of the AR with a long term goal of identifying pathways that can either sensitize AIPCa tumors to known chemotherapeutic agents or that can lead to the development of novel therapeutics.

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References

1. Denis L, Murphy GP. Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer 1993;72(12 Suppl):3888-95.
2. Feldman B, Seidman DS, Levron J, Bider D, Shulman A, Shine S, et al. In vitro fertilization following natural cycles in poor responders. Gynecol Endocrinol 2001;15(5):328-34.
3. Roy AK, Lavrovsky Y, Song CS, Chen S, Jung MH, Velu NK, et al. Regulation of androgen action. Vitam Horm 1999;55:309-52.
4. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. Mol Cell 2002;9(3):601-10.
5. Yeh S, Miyamoto H, Nishimura K, Kang H, Ludlow J, Hsiao P, et al. Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. Biochem Biophys Res Commun 1998;248(2):361-7.
6. Petre-Draviam CE, Cook SL, Burd CJ, Marshall TW, Wetherill YB, Knudsen KE. Specificity of cyclin D1 for androgen receptor regulation. Cancer Res 2003;63(16):4903-13.
7. Yamamoto A, Hashimoto Y, Kohri K, Ogata E, Kato S, Ikeda K, et al. Cyclin E as a coactivator of the androgen receptor. J Cell Biol 2000;150(4):873-80.
8. Palvimo JJ, Reinikainen P, Ikonen T, Kallio PJ, Moilanen A, Janne OA. Mutual transcriptional interference between RelA and androgen receptor. J Biol Chem 1996;271(39):24151-6.
9. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A 2001;98(13):7200-5.
10. Chlenski A, Nakashiro K, Ketels KV, Korovaitseva GI, Oyasu R. Androgen receptor expression in androgen-independent prostate cancer cell lines. Prostate 2001;47(1):66-75.

11. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25(2):276-308.

12. McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 1992;52(24):6940-4.

13. Ratan HL, Gescher A, Steward WP, Mellon JK. ErbB receptors: possible therapeutic targets in prostate cancer? BJU Int 2003;92(9):890-5.

14. Gavrielides MV, Frijhoff AF, Conti CJ, Kazanietz MG. Protein kinase C and prostate carcinogenesis: targeting the cell cycle and apoptotic mechanisms. Curr Drug Targets 2004;5(5):431-43.

15. Yang YM, Bost F, Charbono W, Dean N, McKay R, Rhim JS, et al. C-Jun NH(2)-terminal kinase mediates proliferation and tumor growth of human prostate carcinoma. Clin Cancer Res 2003;9(1):391-401.

16. Edwards J, Krishna NS, Mukherjee R, Bartlett JM. The role of c-Jun and c-Fos expression in androgen-independent prostate cancer. J Pathol 2004;204(2):153-8.

17. Chesire DR, Isaacs WB. Beta-catenin signaling in prostate cancer: an early perspective. Endocr Relat Cancer 2003;10(4):537-60.

18. Libertini SJ, Tepper CG, Guadalupe M, Lu Y, Asmuth DM, Mudryj M. E2F1 expression in LNCaP prostate cancer cells deregulates androgen dependent growth, suppresses differentiation, and enhances apoptosis. Prostate 2006, 66(1): 70-81.

19. Le Page C, Koumakpayi IH, Lessard L, Saad F, Mes-Masson AM. Independent role of phosphoinositol-3-kinase (PI3K) and casein kinase II (CK-2) in EGFR and Her-2-mediated constitutive NF-kappaB activation in prostate cancer cells. Prostate 2005;65(4):306-15.

20. Le Page C, Koumakpayi IH, Lessard L, Mes-Masson AM, Saad F. EGFR and Her-2 regulate the constitutive activation of NF-kappaB in PC-3 prostate cancer cells. Prostate 2005;65(2):130-40.

21. Lessard L, Mes-Masson AM, Lamarre L, Wall L, Lattouf JB, Saad F. NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int 2003;91(4):417-20.

22. Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, et al. Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. Prostate 2002;52(3):183-200.

23. McCarty MF. Targeting multiple signaling pathways as a strategy for managing prostate cancer: multifocal signal modulation therapy. Integr Cancer Ther 2004;3(4):349-80.

24. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3(10):768-80.

25. Vucenik I, Shamsuddin AM. Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic. J Nutr 2003;133(11 Suppl 1):3778S-3784S.

26. Fox CH, Eberl M. Phytic acid (IP6), novel broad spectrum anti-neoplastic agent: a systematic review. Complement Ther Med 2002;10(4):229-34.

27. Agarwal C, Dhanalakshmi S, Singh RP, Agarwal R. Inositol hexaphosphate inhibits constitutive activation of NF- kappa B in androgen-independent human prostate carcinoma DU145 cells. Anticancer Res 2003;23(5A):3855-61.

28. Zi X, Singh RP, Agarwal R. Impairment of erbB1 receptor and fluid-phase endocytosis and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells. Carcinogenesis 2000;21(12):2225-35.

29. Singh RP, Agarwal C, Agarwal R. Inositol hexaphosphate inhibits growth, and induces G1 arrest and apoptotic death of prostate carcinoma DU145 cells: modulation of CDKI-CDK-cyclin and pRb-related protein-E2F complexes. Carcinogenesis 2003;24(3):555-63.

30. Bertrand R, Sarang M, Jenkin J, Kerrigan D, Pommier Y. Differential induction of secondary DNA fragmentation by topoisomerase II inhibitors

in human tumor cell lines with amplified c-myc expression. Cancer Res 1991;51(23 Pt 1):6280-5.

31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.
32. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365-86.
33. Bilbault P, Lavaux T, Lahlou A, Uring-Lambert B, Gaub MP, Ratomponirina C, et al. Transient Bcl-2 gene down-expression in circulating mononuclear cells of severe sepsis patients who died despite appropriate intensive care. Intensive Care Med 2004;30(3):408-15.
34. Tong V, Teng XW, Chang TK, Abbott FS. Valproic acid II: effects on oxidative stress, mitochondrial membrane potential, and cytotoxicity in glutathione-depleted rat hepatocytes. Toxicol Sci 2005;86(2):436-43.
35. Tanaka R, Ariyama H, Qin B, Shibata Y, Takii Y, Kusaba H, et al. Synergistic interaction between oxaliplatin and SN-38 in human gastric cancer cell lines in vitro. Oncol Rep 2005;14(3):683-8.
36. Shah RB, Palamakula A, Khan MA. Cytotoxicity evaluation of enzyme inhibitors and absorption enhancers in Caco-2 cells for oral delivery of salmon calcitonin. J Pharm Sci 2004;93(4):1070-82.
37. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev 2005;11:127-52.
38. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. J Urol 2005;173(2):342-59.
39. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 2. J Urol 2005;173(2):360-72.
40. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol 1997;126(1):59-73.

41. Ferry S, Matsuda M, Yoshida H, Hirata M. Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NFkappaB-mediated cell survival pathway. Carcinogenesis 2002;23(12):2031-41.

42. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 1994;371(6495):346-7.

43. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995;376(6535):37-43.

44. Sun SC, Ganchi PA, Ballard DW, Greene WC. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science 1993;259(5103):1912-5.

45. Westerheide SD, Mayo MW, Anest V, Hanson JL, Baldwin AS, Jr. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Mol Cell Biol 2001;21(24):8428-36.

46. Cha Y, Deisseroth AB. Human interferon regulatory factor 2 gene. Intron-exon organization and functional analysis of 5'-flanking region. J Biol Chem 1994;269(7):5279-87.

47. Dang ZC, Lowik CW. Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway. Mol Cell Biochem 2005;268(1-2):159-67.

48. Rokhlin OW, Taghiyev AF, Guseva NV, Glover RA, Chumakov PM, Kravchenko JE, et al. Androgen regulates apoptosis induced by TNFR family ligands via multiple signaling pathways in LNCaP. Oncogene 2005;24(45):6773-84.

49. Davis R, Jia D, Cinar B, Sikka SC, Moparty K, Zhau HE, et al. Functional androgen receptor confers sensitization of androgen-independent prostate cancer cells to anticancer therapy via caspase activation. Biochem Biophys Res Commun 2003;309(4):937-45.

50. Shamsuddin AM, Yang GY. Inositol hexaphosphate inhibits growth and induces differentiation of PC-3 human prostate cancer cells. Carcinogenesis 1995;16(8):1975-9.

51. Viatour P, Bentires-Alj M, Chariot A, Deregowski V, de Leval L, Merville MP, et al. NF- kappa B2/p100 induces Bcl-2 expression. Leukemia 2003;17(7):1349-56.

52. Dimova DK, Dyson NJ. The E2F transcriptional network: old acquaintances with new faces. Oncogene 2005;24(17):2810-26.

53. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell 1998;1(4):543-51.

54. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2002;2(4):301-10.

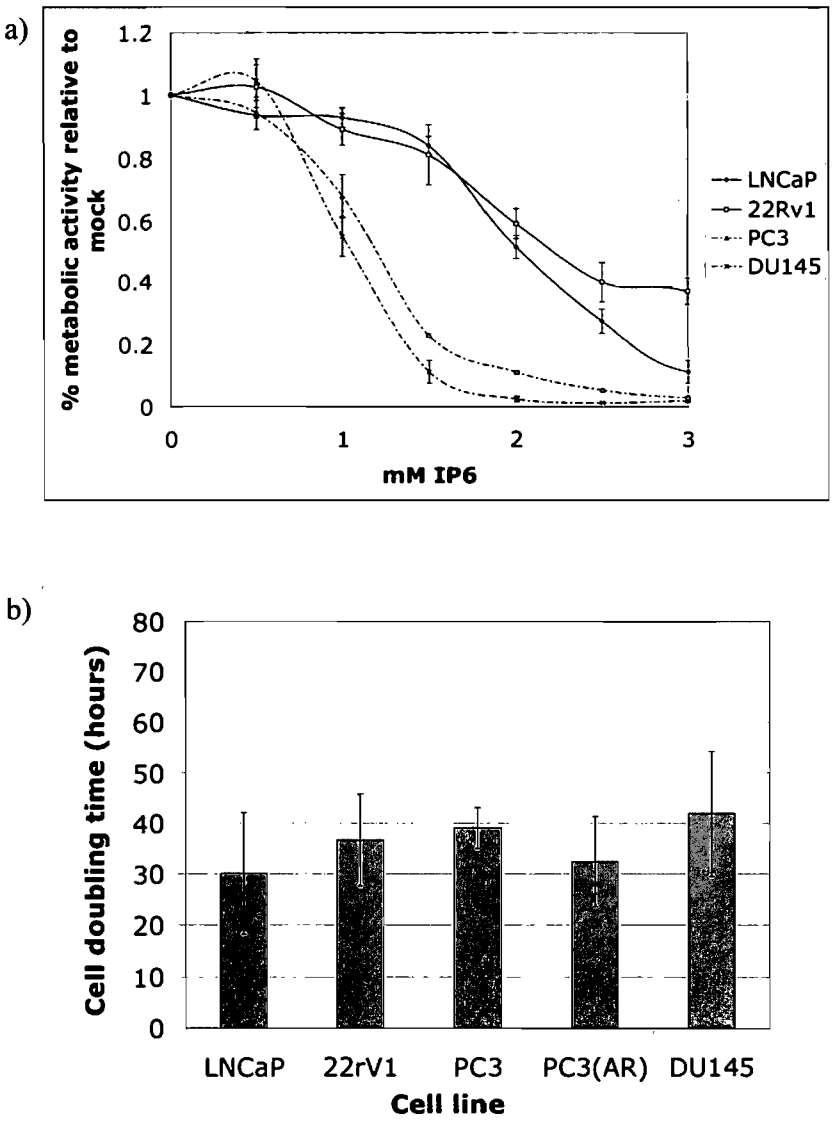

Figure 1. Effect of IP6 on prosate cancer cell lines.

Figure 1. IP6 decreases cellular metabolic activity in PCa cell lines. A) Dashed lines represent hormone insensitive, AR negative, cell lines while solid lines represent hormone sensitive, AR-positive, cell lines. Cells were treated with increasing doses of IP6 for 24 hours and metabolic activity was measured using the WST-1 assay as described in material and methods. Metabolic activity was calculated relative to the mock treated control of each cell line. Values represent an average of 4-7 replicate experiments. Error bars represent standard error of the mean. B) Cell doubling times in normal serum supplemented media. Data represents average of 2 experiments done in triplicate. Error bars represent standard error of the mean. ANOVA analysis revealed no significant differences in doubling times between any of the cell lines ($p>0.05$).

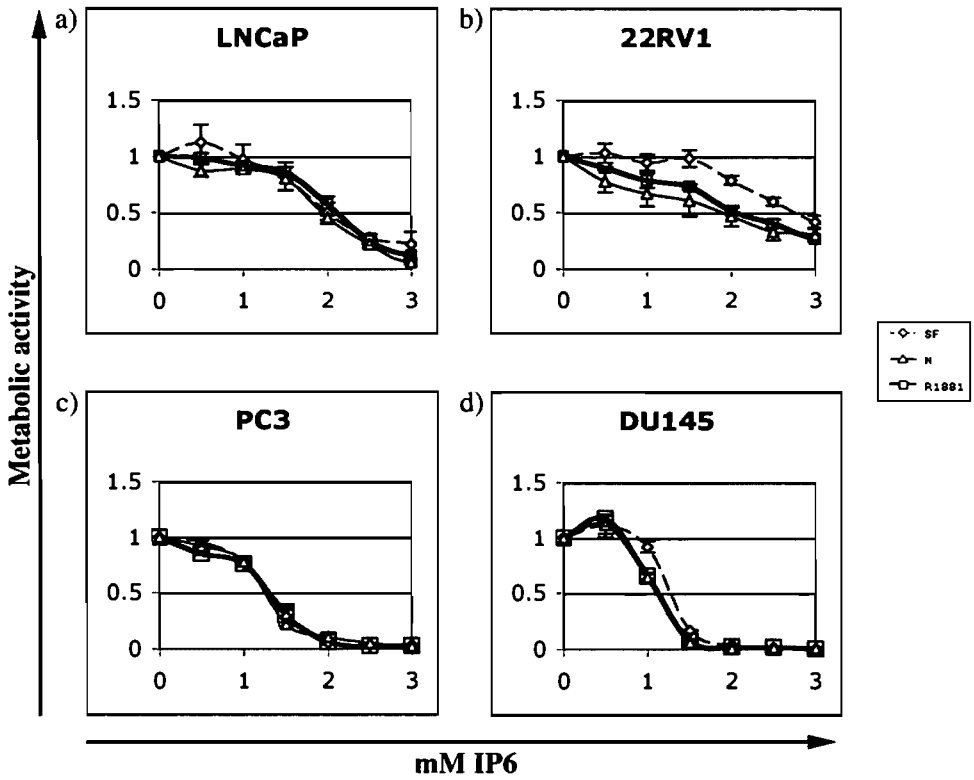

Figure 2. Androgens do not modulate the efficacy of IP6 in prostate cancer cells

Figure 2. Androgens do not modulate the effect of IP6 on PCa cell line metabolic activity. Cells were pre-treated in the respective conditions for 72 hours prior to the assay, which was carried out as described in material and methods. Dashed lines represent steroid free conditions (SF), solid lines are normal serum supplemented conditions (N), and thick lines are normal serum conditions supplemented with 10 nM R1881 synthetic androgen (R1881). Averages were calculated from 2-4 duplicate experiments. Error bars represent standard error of the mean.

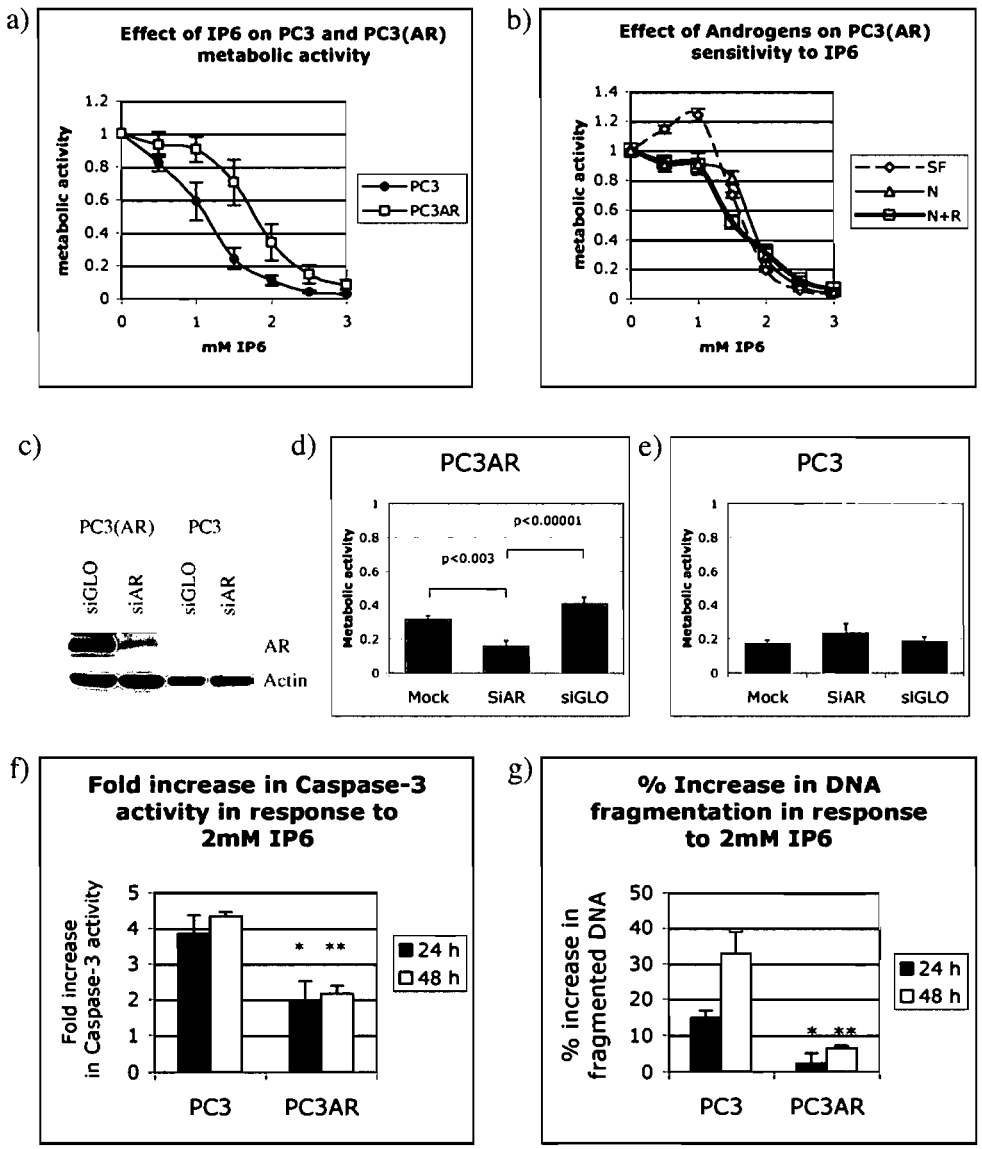

Figure 3. Effect of AR expression on the efficacy of IP6 in PC3 cells.

Figure 3. Effect of stable expression of the AR in PC3 cells. a) AR expression in PC3 cells protects from the effects of IP6 on cell metabolism. WST-1 assays were performed in normal serum complemented media as described in material and methods. Data represents average of 3 experiments done in triplicate. Differences between PC3 and PC3(AR) cells were significant at all concentrations ($p<0.05$). **b)** Protective effect of the AR is not androgen-dependent. WST-1 assay was performed as described in Figure 2. Data represents average of 2-3 duplicate experiments. SF= steroid free culture medium, N= normal serum complemented culture medium, N+R= Normal serum-complemented culture medium with 10nM R1881. **c)** SiRNA was transfected as described in material in methods. 72 hours following transfection, 2mM IP6 was added. WST-1 assays were performed 24 hours later. Protein was extracted from the same cells as those used in WST-1 assays 24 hours after IP6 treatment (mock-treated). Western blotting demonstrates that the siRNA targeting the AR effectively decreased AR expression in PC3(AR) cells compared to siGLO transfection control. **d-e)** An siRNA targeting the AR sensitizes PC3(AR) but not PC3 cells to the effects of IP6. Data represents metabolic activity as measured by WST-1 relative to mock-treated control of PC3(AR) (**d**) and PC3 (**e**) cells after 24 hour treatment with IP6. Mock= mock-transfected cells; siAR=cells transfected with AR-targeting siRNA+ siGLO RISC-free transfection control siRNA; siGLO= RISC-free transfection control siRNA. Data represents average of 3 triplicate experiments. All Error bars are standard error of the mean. Indicated p values were calculated using student's T-test. Differences were also significant using ANOVA ($p<0.001$). **f)** IP6 induces caspase-3-like activation in PC3 cells but to a lesser extent in PC3(AR) cells. The caspase-3 assay was performed as described in materials and methods after a 24 or 48-hour treatment with 2 mM IP6. Data represents fold change in caspase-3-like activity as compared to mock-treated control. Fold changes were calculated from 4-8 replicates. *$p<0.001$, **$p<0.001$. **g)** IP6 increases DNA fragmentation to a lesser extent in PC3(AR) cells than in PC3 cells. Filter elution assays were performed as described in material and methods using 2 mM IP6 after 24 or 48 hours. Data represents average % increase in DNA fragmentation as compared to

mock-treated control. % Increase in fragmented DNA was calculated using formulas described in material and methods from 3 independent duplicate experiments. *$p<0.01$, **$p<0.05$.

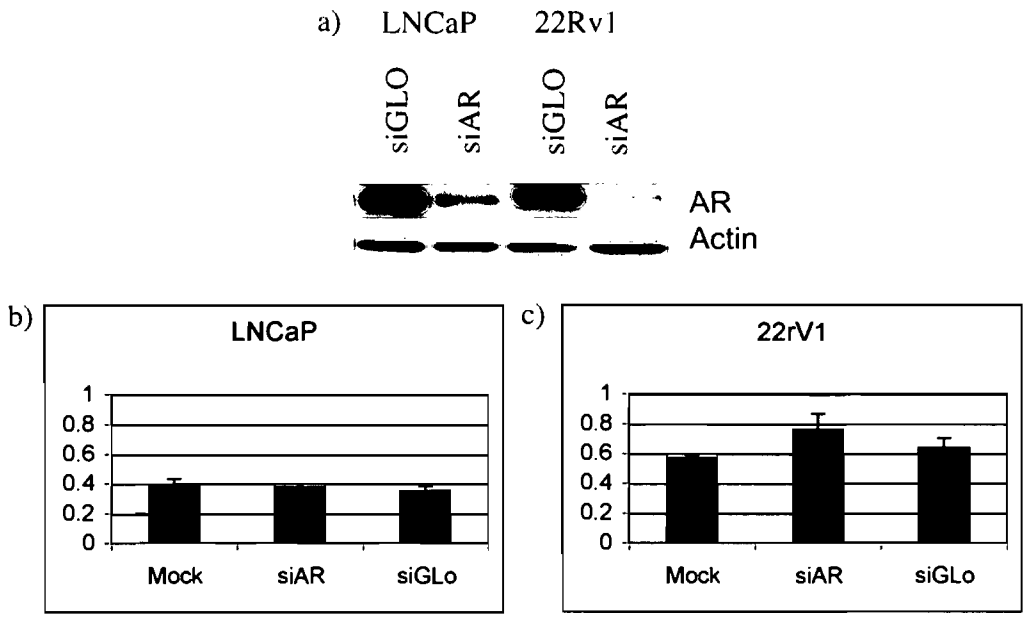

Figure 4. AR down-regulation does not modulate the efficacy of IP6 in prostate cancer cells endogenously expressing the AR.

Figure 4. No effect of AR-targeting siRNA on LNCaP and 22Rv1 cells on IP6 efficacy. A) SiRNA was transfected as described in material in methods and cells were treated as described in figure 3. Western blotting demonstrates that the siRNA targeting the AR effectively decreased AR expression in LNCaP and 22Rv1 cells compared to siGLO transfection control. b-c) AR-targeting siRNA does not sensitize LNCaP or 22Rv1 cells to the effects of IP6. Data represents metabolic activity as measured by WST-1 relative to mock-treated control of LNCaP (b) and PC3 (c) cells after 24-hour treatment with IP6. Mock= mock-transfected cells; siAR=cells transfected with AR-targeting siRNA+ siGLO RISC-free transfection control siRNA; siGLO= RISC-free transfection control siRNA. Data represents average of 2-3 triplicate experiments. All Error bars are standard error of the mean. No statistically significant differences were revealed by T-Test or ANOVA.

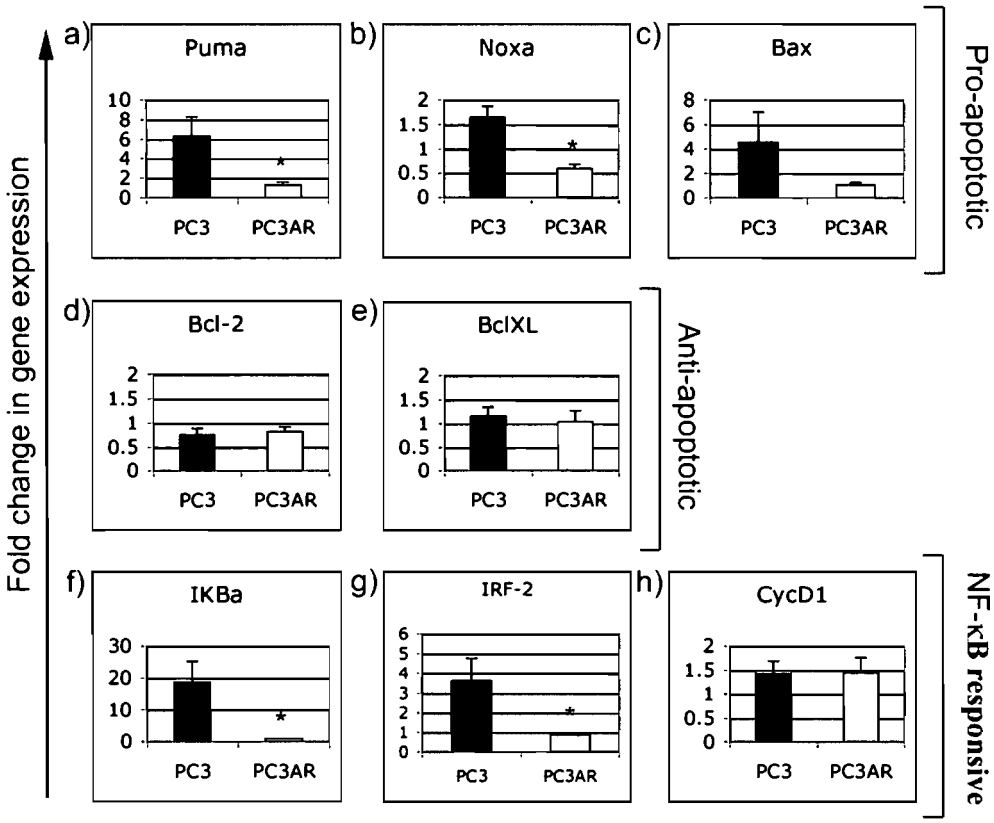

Figure 5. Effect of IP6 on the expression of NF-κB-responsive genes and of genes involved in apoptosis

Figure 5. IP6 induces the expression of NF-κB-dependent and p53/E2F-regulated genes in PC3 but not in PC3(AR) cells. Cells were treated with 1mM IP6 for 24 hours as described in materials and methods. Following RNA extraction and cDNA synthesis, Real-time PCR was performed and gene expression (relative to actin B) was calculated using the method described by Pfaffl et al. a-c) pro-apoptotic protein-encoding genes puma (a), noxa (b) and bax (c). d-e) Anti-apoptotic protein-encoding genes bcl-2 (d) and bcl-X_L (e). f-h) NF-κB-responsive genes IκB-α (f), IRF-2 (g) and cyclin D1 (h). Data represents fold change in gene expression from mock-treated control to IP6-treated samples. Averages were calculated from 2 independent duplicate experiments. Error bars represent standard error of the mean. Differences between fold induction of gene expression by IP6 in PC3 and PC3(AR) cells were significant for puma ($p<0.002$), noxa ($p<0.001$), IRF-2 ($p<0.01$) and IκB-α ($p<0.0001$).

CHAPTER III

NOXA and PUMA Expression Add to Clinical Markers in Predicting Biochemical Recurrence of Prostate Cancer Patients in a Survival Tree Model

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Author contributions:

Although all authors played a role in editing the paper, I wrote the manuscript and did most of the work presented. Abdulhadi Aldejmah helped with immunohistochemistry experiments and evaluation of the stained tissue arrays. Ali Filali Mouhim played an integral role in the survival tree analyses. Benjamin Péant helped with western blotting experiments. Mona Alam Fahmy, Hervé Koumakpayi, Kaniska Sircar and Louis R. Bégin were pivotal in the construction of the tissue arrays.

Key words: NOXA, PUMA, Biochemical recurrence, Prostate cancer, Survival trees

Abstract

Purpose: To assess the expression of pro-apoptotic NOXA and PUMA in prostate tissues and delineate their association with prostate cancer (PCa) recurrence.

Experimental Design: Normal, prostatic intraepithelial neoplasia (PIN), hormone-sensitive (HS) prostate cancer (PCa), and hormone-refractory (HR) PCa tissues were used to build tissue microarrays encompassing a total of 135 patients. Two observers assessed the intensity of NOXA and PUMA immunohistochemical staining using a composite color scale. 180 Recursive Partitioning And Regression Tree (RPART) models were generated to predict biochemical recurrence (BCR) within HS cancer patients using NOXA, PUMA and clinical parameters. Models were then ranked according to the Integrated Brier Score (IBS).

Results: Increasing NOXA expression was associated with PCa progression reaching the highest levels in HR PCa. Increased NOXA expression was observed in 68% of HS cancer patients and was predictive of BCR (LR=8.64 p=0.003). In contrast, PUMA expression was highest in HS cancer and although 70% of HS cancer patients exhibited increased PUMA expression, PUMA alone could not predict the onset of BCR. Interestingly, the top-ranking RPART model generated (IBS=0.107; 95% CI=0.065-0.128) included surgical margin status, NOXA and PUMA expression although recurrent prognostic classification schemes obtained in the top ten models favored a survival tree model containing margin status, NOXA expression and pre-operative prostate specific antigen (PSA) (IBS=0.114; 95% CI=0.069-0.142).

Conclusion: We conclude that NOXA and PUMA expression may be linked to PCa progression and propose further validation of a survival tree model including surgical margin status, NOXA expression, and pre-operative PSA for predicting BCR.

Introduction

Prostate cancer (PCa) remains a leading cause of cancer-related death in North American men (1). Although localized forms of the disease can often be successfully treated by surgery or radiotherapy, a significant proportion of patients having undergone such interventions are at risk of disease relapse. For this reason, considerable efforts have been made in order to discover new molecular markers that can accurately predict the onset of disease relapse and lead to better-targeted and more effective treatment.

Androgen deprivation therapy is often used to treat recurrent PCa and can increase patient survival; however, this form of therapy eventually gives rise to androgen-independent PCa (or AIPCa) (2, 3). Because the treatment of AIPCa remains palliative to date (4-6), much effort has been devoted to describing the molecular mechanisms associated with the transition of androgen-dependent PCa to an androgen-independent state. Many studies have established a role for androgen receptor (AR) signaling in this phenomenon (7, 8). However, increasing evidence suggests that other signaling pathways may also be important for progression to an androgen-independent state (9-14). At the convergence of many of these pathways, it has been suggested that PCa cells can become resistant to treatment-induced apoptosis through the up-regulation of anti-apoptotic proteins such as BCL-2, BCL-X_L and MCL-1 (15, 16). Several studies have detected up-regulated BCL-2, BCL-X, and MCL-1 expression in high-grade PCa tumors and in AIPCa (17-21).

In theory, enhanced resistance to apoptosis can also be achieved by the down-regulation of pro-apoptotic proteins (22). To date, few studies have looked at the expression of pro-apoptotic proteins in PCa. So far, most studies addressing this question have focused on BAX, a pro-apoptotic protein that elicits its effect at the level of the mitochondrial outer membrane where it promotes mitochondrial depolarization, a key event in the intrinsic apoptotic pathway. Although it is clear from several immunohistochemistry (IHC) studies that BAX is expressed in the

large majority of tumors, the association between BAX expression and PCa progression remains uncertain (18, 23-26).

NOXA and PUMA are two BH3-only pro-apoptotic proteins that act upstream of BAX/BAK in order to promote mitochondrial depolarization. NOXA is essentially thought to sensitize cells to the action of activator BH3-only pro-apoptotic proteins by disrupting their interaction with anti-apoptotic proteins. Recent evidence suggests that NOXA specifically disrupts the interaction of MCL-1 with activator BH3-only proteins BID, BIM and PUMA (27). In turn, activator BH3-only proteins such as PUMA and BID interact with the Hα1 helix of BAX to induce conformational changes leading to permeation of the mitochondrial outer membrane (28).

To date, few IHC studies have looked at PUMA or NOXA expression in cancer. In melanoma, weak PUMA expression was linked to poor patient survival (29) particularly in patients also showing elevated levels of phosphorylated AKT (30). In colorectal cancer, no relationship with clinical outcome was found although 29% of tumors overexpressed PUMA (as opposed to 4% showing decreased expression) (31). Similarly, NOXA expression was increased in 16% of colorectal tumors but was not associated with disease outcome (32). To date, neither NOXA nor PUMA has been studied in relation to PCa progression and clinical outcome.

To assist in the process of prognostic marker discovery, increasingly powerful statistical methods are being developed and applied. Of these methods, survival trees are particularly attractive when looking at multiple markers within one or more signaling pathways. Survival tree algorithms are based on recursive partitioning of the covariate space (33, 34) and their graphical output facilitates the visualization of prognostic groups reflecting multi-marker interactions. In this study, we looked at the expression of NOXA and PUMA using tissue micro-arrays containing normal prostate tissue, primary PCa and its adjacent non-neoplasic tissue, as well as specimens of androgen-independent PCa, representing a total of 135 patients. We then used survival trees to evaluate the ability of

NOXA and PUMA, alone or in combination with clinical markers, to predict the onset of biochemical recurrence in patients presenting primary PCa.

Materials and Methods

Patient cohort

A total of 51 normal prostate specimens were obtained from cancer-free patients. An additional 64 paraffin-embedded human primary PCa specimens from patients who had undergone radical prostatectomy between 1993 and 2000 were also used. Futhermore, trans-urethral resections of the prostate (TURP) specimens from 30 AIPCa patients were obtained. Regions of non-neoplasic and cancerous epithelial tissue were identified by two pathologists and subsequently spotted on tissue micro-arrays. In the sub-cohort of 64 primary PCa tumors, which was used for retrospective prognostic studies, no patient received preoperative hormone therapy and all cases had a clinical follow-up of at least 5 years or until death (average follow up of 72 months). No age difference was observed between the group of patients who relapsed and the group that did not. Postoperative PSA was available for all patients. The time to biochemical recurrence (BCR) was defined as the time elapsed between the date of surgery and the date where PSA first increased from undetectable levels to above 0.3 ng/ml and rising, consistent with previous studies (35-37). Non-relapsed patients had a PSA remaining below 0.3 ng/ml after radical prostatectomy. For PCa specimens, the final staging, grading and histo-pathological diagnosis was based on the pathology report in agreement with the review from an independent pathologist. Specimens were obtained from consenting patients and the institutional ethics review committee approved the study.

Tissue array construction and verification

Tissue arrays containing a total of 613 one millimeter-wide cores of prostate tissues were built and used for IHC studies. For the sub-cohort containing normal tissue cores obtained from 51 autopsied patients, two cores per patient were spotted on a tissue micro-array. For the prognostic sub-cohort of primary

tumors, two non-neoplastic and four cancerous cores per patient were spotted on tissue arrays. For the hormone-refractory TURP sub-cohort, four cores per patient were included on a tissue microarray. Following tissue microarray construction, 4 μm thick cross-sections were put on glass slides and stained with hematoxylin & eosin as well as for cytokeratin 34βE12 and reviewed by two pathologists. All cores were subsequently re-categorized as containing no epithelial cells, non-neoplasic epithelium, focal atrophy, PIN, or adenocarcinoma. Cores containing no epithelial cells or focal atrophy were not considered in the analysis. Following reclassification, the final specimen cohort consisted of 601 cores representing 43 patients with normal prostate tissues, 62 patients presenting primary PCa tissues and 30 patients with hormone-refractory PCa for a total of 135 patients. Patient characteristics are summarized in Table 1.

Protein extraction

Confluent LNCaP, 22Rv1, PC3 and DU145 cells were scraped, washed twice with cold PBS and pellets were frozen at –80°C. Subsequently, whole cell extractions were performed by applying cold lysis buffer (10 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 0.5% NP-40, 0.5 mM PMSF, 0.2 mM orthovanadate, 2 mg/ml of aprotinin, leupeptin and pepstatin) on ice for 30 min. Whole cell extracts were collected after centrifugation in a Heraeus Biofuge (13,000 rpm for 10 min at 4°C) and were immediately stored at -80°C. Protein concentration was measured by Bradford assays (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instructions.

Western blot analysis:

For Western blot analysis, 50 μg of whole cell protein extract were resolved on a 12.5% polyacrylamide gel and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Blots were blocked using 5% non-fat dry milk in PBS-Tween 0,05% buffer overnight at 4°C and probed using either a monoclonal antibody raised against recombinant GST-tagged full-length NOXA (OP180, Calbiochem, La Jolla, CA), polyclonal antibody raised against amino

acids 2-16 of PUMA (PC686, Calbiochem, La Jolla CA) or actin B (ab6276-100, Abcam, Cambridge, UK) for 1 hour at room temperature in blocking buffer (1:500). Membranes were then incubated with secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences Inc., Arlington Heights, IL) in blocking buffer for 1 hour at room temperature and developed with enhanced chemiluminescence (ECL) substrate (Amersham Life Sciences Inc., Arlington Heights, IL).

Immunohistochemistry

Samples were immunostained with either anti-NOXA antibody (OP180) at 50 ng/μl or anti-PUMA antibody (PC686) at 4 ng/μl diluted in PBS. Primary antibody detection was done using the LSAB 2 peroxidase system from Dako Diagnostics Inc (CA, USA). Staining was performed as described previously (35, 38-40). Briefly, tissue samples were deparaffinized, rehydrated, and treated with 0.3% H_2O_2 to eliminate endogenous peroxidase activity. An antigen retrieval step was performed using 10 mM citrate buffer (pH 6.0) applied for 17.5 minutes at 95°C. All following steps were performed at room temperature. The sections were blocked with a protein-blocking serum-free reagent (Dako) and incubated with primary antibody for 60 minutes, followed by a 20 minute treatment with the secondary biotinylated antibody (Dako), washed 5 minutes in PBS, and then incubated for 20 minutes with streptavidin-peroxidase (Dako). Following an additional 5 minute PBS wash, reaction products were developed with diaminobenzidine (Dako) containing 0.3% H_2O_2 as a substrate for peroxidase. Nuclei were counterstained with Harris hematoxylin (Sigma-Aldrich, MO, USA). No non-specific secondary antibody staining was observed when PBS was used instead of the primary antibody.

Scoring procedure

For NOXA and PUMA-stained tissues, digital pictures were taken of each core on an Olympus BX51 microscope using Q capture imaging software (Olympus, London, UK). Two independent observers quantified epithelial

staining intensity using a color scale (Fig.2h) constructed from the various staining intensities observable in the digital pictures using the eyedropper tool in Adobe Photoshop 7.0. The observers assessed the percentage of epithelial cells representing each color of the scale (0-9) and an overall score was calculated from the sum of the products derived from percentage (0-100%) multiplied by the scale value (0-9) for each core. Hence, all staining intensity values are on a continuous scale of 0 to 9. Notably, intra-class correlation coefficients (ICC) (a measure of reliability between the two observers) were found to be excellent using this method (ICC>0.75, (41)), in sharp contrast with initial estimates done using conventional microscopy methods (ICC<0.5). Overall intensity values from each observer, obtained using the digital pictures, were then averaged and used for further statistical analyses.

Statistics

Mean staining intensities of cores from cancer-free patients, of non-neoplasic, PIN and cancer cores from hormone-sensitive PCa patients as well as of cancer TURP cores from hormone-refractory PCa patients were calculated. Kruskal-Wallis non-parametric tests were used to assess statistical significance of observed differences in mean staining intensity. All correlation coefficients were computed using Spearman's non-parametric test. Cut-off determination and survival tree construction was done using the RPART (Recursive PARTitioning) libraries (33), which extends the CART (Classification and Regression Trees) routine (34). Model accuracy was assessed using the integrated Brier score for censored data (IBS) (42). We used 200 bootstrap (43) samples to compute the 95% confidence interval (CI) on the IBS. Survival tree growth was controlled using the minimum splitting (*minsplit*) criterion implemented in RPART. This parameter controls the minimum number of observations that must exist in a node, in order for a split to be attempted. For combination models including NOXA and/or PUMA as well as combinations of the four clinical markers, all possible RPART models were generated using three different values of *minsplit* (20, 25 and 30). This generated 180 combinations corresponding to 69 different

unique tree models, which were ranked according to IBS. Kruskal-Wallis, Spearman, and Kaplan-Meier analyses were performed using Statistical Package for the Social Sciences (SPSS) version 11 (SPSS, Inc., Chicago, USA). Tree building and the calculation of IBS were carried out in the R (version 2.4.0; (44)) system for statistical computing (www.cran.r-project.org), using rpart and ipred packages respectively.

Results

NOXA and PUMA Expression in Prostate Cancer Cell Lines

We used western blotting on whole cell extracts in order to assess the expression of NOXA and PUMA proteins in PCa cell lines. As shown in figure 1, the antibodies targeting NOXA and PUMA detected the expected ~6 kD and 23 kD bands (respectively) and revealed variable but apparent NOXA and PUMA expression in all PCa cell lines. For PUMA, cell line expression levels were found to be highest in 22Rv1 followed by LNCaP and PC3 cells, with DU145 exhibiting the lowest PUMA expression. NOXA expression levels were highest in DU145 cells, followed by PC3 and 22Rv1 cells, with lowest expression levels in LNCaP cells.

NOXA Expression in Prostate Tissue Sub-types

To determine whether NOXA expression could be linked to PCa progression, we stained prostate tissue micro-arrays using the antibody recognizing NOXA (same as used in Fig.1). In general, we found that this antibody stained the cytoplasm of epithelial cells (Fig.2 a, c, e). In many normal cores from cancer-free patients and non-neoplastic cores found adjacent to cancer (hereby referred to as NA), we observed more intense staining in the basal cell layer of epithelial glands (Fig. 2a). To increase interobserver reliability and facilitate retrospective interpretation of the results obtained, we used a standard color scale (Fig.2h) constructed from digital pictures of tissue cores as described in materials and methods. Overall, we found that cores taken from normal patients expressed

significantly less NOXA than all other tissue sub-types obtained from PCa patients, including NA cores (Fig 3a). We also observed a slight but statistically insignificant decrease in PIN as opposed to NA cores ($p=0.09$). Although hormone-sensitive (HS) cancer tissues exhibited higher mean NOXA expression than both NA and PIN cores, ($p<0.001$), hormone-refractory (HR) TURP specimens exhibited the highest mean NOXA staining (mean=5.09, $p<0.001$). Notably, in the subgroup of patients for which we had both NA and HS cores ($n=51$), 68% exhibited increased NOXA expression in HS cores.

PUMA Expression in Prostate Tissue Sub-types

Similarly to what was observed with the anti-NOXA antibody, we found that the antibody targeted against PUMA (same as used in Fig.1) generally stained the cytoplasm of epithelial cells (Fig 2b, d, f). Basal cell staining was also apparent in several normal prostate cores as well as in NA cores (Fig 2b). PUMA staining was subsequently evaluated using the same method employed for NOXA and mean core intensity was calculated for each core sub-type. As shown in figure 3b, we observed a significant increase in PUMA expression in NA cores as compared to normal prostate cores ($p<0.001$). While PUMA expression was similar in NA and PIN cores, HS cancer cores exhibited significantly higher PUMA expression than both NA/PIN ($p<0.001$). It should be noted however that mean PUMA expression in HR cores was not found to be significantly different from that observed in NA cores ($p=0.654$). Similarly to what was observed for NOXA, in the subgroup of patients for which we had both NA and HS cancer cores ($n=51$), 70% exhibited increased PUMA expression in HS cancer.

NOXA and PUMA Expression Can Predict the Onset of Biochemical Recurrence

Pro-apoptotic proteins such as PUMA and NOXA play a role in the initiation of cell death to various cellular stresses. Hence, we wondered whether NOXA and/or PUMA expression could be predictive of PCa re-emergence following radical prostatectomy. To address this question, we used BCR as a

surrogate endpoint and determined whether NOXA and/or PUMA expression could be predictive of BCR. We employed the rpart function in R to assess whether NOXA or PUMA status alone could stratify patients in function of BCR onset within the sub-cohort of 62 patients presenting HS cancer. Using optimal cut-offs obtained by rpart, corresponding to the primary splitter of the root node, we found that high NOXA expression ($\geq$5.5 on a scale of 0-9) was associated with an earlier and more frequent onset of BCR (Log Rank or LR=8.6 p=0.003; Fig. 4a). On the other hand, PUMA expression alone was not significantly predictive of the onset of BCR (LR=2.5 p=0.114; Fig. 4b). Interestingly, including both NOXA and PUMA in the RPART model revealed that low PUMA expression was associated to more rapid progression towards BCR but specifically when NOXA expression was also low (LR=15.6 $p<5x10^{-4}$, Fig 4c, Table 2). In this model, patients exhibiting high NOXA ($\geq$5.5) expression were most likely to quickly undergo BCR, with approximately 77% (10/13) of these patients having undergone relapse before 3 years. Within the group of patients expressing low levels of NOXA, low expression of PUMA (<6.6) was associated to earlier and more frequent onset of BCR with close to 46% (16/35) of patients having relapsed within 3 years. In contrast, patients exhibiting both low NOXA and high PUMA infrequently underwent BCR, with only 14% (2/14) having undergone relapse at 3 years. Because of potential over-fitting due to the application of cut-offs obtained by RPART from the same test data set, Kaplan-Meier plots and associated LR p-values should be considered as purely descriptive measures since the survival outcomes were used to define the prognostic groups.

NOXA and PUMA Expression Predict the Onset of BCR in Combination with Clinical Markers

We next wondered whether NOXA and PUMA expression could help to predict BCR in combination with other clinico-pathological parameters such as pre-operative PSA, Gleason score, pathological stage and resection margin status. We thus generated several RPART models by using as input variables all possible combinations of the four clinical markers with NOXA and/or PUMA and ranked

them according to the IBS, where lower IBS means greater accuracy. The top-ranking model stratified patients first on the basis of margin status (negative=good prognosis) then on the basis of NOXA within negative margins (NOXA<5.2=good prognosis) and on the basis of PUMA within the positive margins (PUMA<6.1=good prognosis). Furthermore, PUMA also stratified negative margin patients expressing low levels of NOXA, with high PUMA expression (PUMA≥6.1) being surprisingly associated with good prognosis (0/13 patients relapsed, Fig 5a). This model had an IBS of 0.107 (95% CI=0.065-0.128, table 2). Interestingly, we found that nine of the top ten RPART models exhibited an initial stratification according to resection margin status followed by that of NOXA expression in negative margins (Fig 5b). In contrast, only three of the top ten models included PUMA. In seven of the top ten models, positive margin patients as well as negative margin patients with low NOXA expression could be further stratified by pre-operative PSA and/or Gleason score where patients exhibiting low pre-operative PSA or low Gleason had a better prognosis. Notably, in all of these seven models the best prognostic group (negative margin, low NOXA expression and low pre-operative PSA or Gleason) did not relapse. In one of these models with an IBS of 0.114 (95% CI=0.069-0.142, table 2), 0/9 patients exhibiting negative margins, low NOXA expression and low pre-operative PSA (<6.5) relapsed in the best prognostic group (Fig 5c). In contrast, the top model that included only margin status and pre-operative PSA had an IBS of 0.135 (95% CI=0.093-0.157, table 2) where 3/20 patients relapsed in the best prognostic group. Of the RPART models composed exclusively of clinical markers, the top-ranking model was one that included margin status and Gleason score and had an IBS of 0.132 (95% CI=0.090-0.160, table 2).

Discussion

To our knowledge this is the first study describing the expression of NOXA or PUMA in a cohort of patients representing various histopathological sub-types of PCa. In our overall cohort of 135 patients we found that mean NOXA expression increased gradually going from normal prostate cores to NA

and PIN cores, followed by HS cancer cores and finally to HR cancer cores, the latter expressing the highest levels of NOXA (Fig 2a). These data suggest that increasing NOXA expression may be associated to PCa progression. In contrast with what has been previously observed in colorectal cancer (32), we found that increased NOXA expression is a frequent occurrence in PCa (16% in colorectal cancer vs. 68% of PCa patients). In addition, we found that NOXA expression is associated with clinical outcome, which was not observed in the colorectal cancer study. In contrast with NOXA, the association between PCa progression and PUMA appears to be more complex. Although we found that 70% of HS patients showed elevated PUMA (as opposed to 29% in melanoma (31)), our data suggest that PUMA expression does not further increase in HR PCa.

These findings are somewhat reflected in what was observed in PCa cell lines using western blots probing for NOXA and PUMA expression. In Figure 1, we can see that taken together, the two androgen-insensitive cell lines PC3 and DU145 express relatively higher levels of NOXA as compared to androgen-responsive LNCaP and 22Rv1 cells. In contrast, LNCaP and 22Rv1 cells appear to exhibit higher levels of PUMA. These findings are somewhat surprising because NOXA and PUMA have been found to have more than one transcriptional regulator in common, including p53 and E2F1 (45). However, we found that within cores there was a generally strong correlation between PUMA and NOXA expression (Spearman Coefficient = 0.586; $p<10^{-6}$; data not shown). Altogether, these data may be indicative of the involvement of molecular pathways that lead to "de-coupled" PUMA/NOXA in HRPCa. Further investigation will be required to address this possibility.

To date, investigators have typically used linear Cox proportional hazard models to stratify patients' risk with respect to the expression of molecular markers. However, Cox models neither handle complex interactions among prognostic factors efficiently, nor take into account nonlinear effects (46, 47). To overcome these limitations, tree–based methods offer an attractive alternative to Cox models (48). In this study, we used survival trees to assess the ability of NOXA and PUMA to predict BCR alone and in combination with clinical

markers. Although this method is increasingly used for immunohistochemical analyses in cancer, its specific application to PCa cohorts has been thus far limited and generally focused on existing clinical parameters (49-52). However, one PCa study has recently applied the survival tree method to assess the prognostic ability of alpha-methylacyl CoA racemase detected by immunohistochemistry as was done here for PUMA and NOXA (53).

Assessing the predictive performance measure and model selection criteria for prognostic models remains a matter of debate. For survival tree-based methods, the IBS is currently thought to be the most appropriate index (42, 54). Using IBS, we determined that the most accurate model for predicting BCR in our cohort was one that included surgical margin status, NOXA, and PUMA expression (IBS=0.107). Although this model was particularly good at predicting which patients would not undergo BCR (0/13 in the best prognostic group, fig 5a-b), it presented a complex behavior of PUMA wherein its effect on BCR onset in negative margins was opposite to that found in positive margins. Because PUMA expression alone could not significantly predict BCR as shown in Kaplan Meier analyses (Fig 4b), it is unclear whether PUMA expression truly holds valuable clinical information. On the other hand, NOXA was a significant predictor of BCR when assessed alone (Fig 4a) and was incorporated into the top nine RPART models where low NOXA expression was consistently associated with good prognosis. For this reason as well as for practical considerations pertaining to some degree of subjectivity in the Gleason grading, we favor the model presented in Fig 5d-e, including NOXA expression, surgical margin status and pre-operative PSA, for future evaluation.

One limitation in the present study is the relatively small size of our prognostic cohort (n=62), which may affect the reliability of the RPART algorithm. Nonetheless, we could observe recurrent and stable tree structures that were consistently present in the top nine models. Another relevant concern is that re-applying a RPART model established from one data set onto the same data set can lead to over-fitting. To account for this, we used bootstrap re-sampling to calculate 95% confidence intervals for the IBS as has been done elsewhere (55).

Nonetheless, the results obtained here remain of an exploratory nature and will require subsequent external validation in another cohort. In addition, it will be of significant interest to determine whether NOXA/PUMA expression can also be useful prognostic markers at the biopsy level. In either case, the color scale method for measuring staining intensity devised here will likely be useful for the evaluation and classification of future samples.

Overall, our findings are somewhat at odds with the roles of NOXA and PUMA as pro-apoptotic factors. While some have found that increasing apoptotic index is associated with disease recurrence (56) others have detected decreasing apoptosis in PCa progression (57). One possible reason for these discrepancies is that increased apoptosis may be counterbalanced by increased cell proliferation, leading to more rapid cellular turnover. Notably, others have found that increasing expression of the cell proliferation marker Ki67 correlates with Gleason grade and with decreased PCa patient survival (58, 59). Although we are currently investigating whether this is the case for our patient cohort (Gannon P.O. et al, manuscript in preparation), it is important to note that we did not find a significant correlation between Gleason score and either NOXA or PUMA in the present study (data not shown). As such, another possibility is that increased NOXA/PUMA expression is an indirect result of de-regulated activation of factors mediating NOXA/PUMA transcription or protein stabilization. Potential candidates for this are varied and include p53, E2F as well as forkhead family transcription factors (45, 60, 61).

Conclusion

We conclude that NOXA and PUMA expression may be linked to PCa progression. We also suggest that the assessment of NOXA expression may be particularly useful for PCa prognosis, as it may extend the ability of existing clinical markers to predict BCR. We believe that a survival tree model including NOXA, surgical margin status and pre-operative PSA status deserves external validation in a larger cohort.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2007. CA Cancer J Clin, 2007; 57: 43-66.

2. Landis SH, Murray T, Bolden S, and Wingo PA. Cancer statistics, 1999. CA Cancer J Clin, 1999; 49: 8-31, 1.

3. Denis L and Murphy GP. Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer, 1993; 72: 3888-95.

4. Culine S and Droz JP. Chemotherapy in advanced androgen-independent prostate cancer 1990-1999: a decade of progress? Ann Oncol, 2000; 11: 1523-30.

5. Crawford ED, Eisenberger MA, McLeod DG, et al. A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. N Engl J Med, 1989; 321: 419-24.

6. Morris MJ and Scher HI. Novel strategies and therapeutics for the treatment of prostate carcinoma. Cancer, 2000; 89: 1329-48.

7. Coughlin SS and Hall IJ. A review of genetic polymorphisms and prostate cancer risk. Ann Epidemiol, 2002; 12: 182-96.

8. Shang Y, Myers M, and Brown M. Formation of the androgen receptor transcription complex. Mol Cell, 2002; 9: 601-10.

9. Ratan HL, Gescher A, Steward WP, and Mellon JK. ErbB receptors: possible therapeutic targets in prostate cancer? BJU Int, 2003; 92: 890-5.

10. Gavrielides MV, Frijhoff AF, Conti CJ, and Kazanietz MG. Protein kinase C and prostate carcinogenesis: targeting the cell cycle and apoptotic mechanisms. Curr Drug Targets, 2004; 5: 431-43.

11. Yang YM, Bost F, Charbono W, et al. C-Jun NH(2)-terminal kinase mediates proliferation and tumor growth of human prostate carcinoma. Clin Cancer Res, 2003; 9: 391-401.

12. Edwards J, Krishna NS, Mukherjee R, and Bartlett JM. The role of c-Jun and c-Fos expression in androgen-independent prostate cancer. J Pathol, 2004; 204: 153-8.

13. Chesire DR and Isaacs WB. Beta-catenin signaling in prostate cancer: an early perspective. Endocr Relat Cancer, 2003; 10: 537-60.

14. Libertini SJ, Tepper CG, Guadalupe M, et al. E2F1 expression in LNCaP prostate cancer cells deregulates androgen dependent growth, suppresses differentiation, and enhances apoptosis. Prostate, 2006; 66(1):70-81

15. Catz SD and Johnson JL. BCL-2 in prostate cancer: a minireview. Apoptosis, 2003; 8: 29-37.

16. Tantivejkul K, Loberg RD, Mawocha SC, et al. PAR1-mediated NFkappaB activation promotes survival of prostate cancer cells through a Bcl-xL-dependent mechanism. J Cell Biochem, 2005; 96: 641-52.

17. Zellweger T, Ninck C, Bloch M, et al. Expression patterns of potential therapeutic targets in prostate cancer. Int J Cancer, 2005; 113: 619-28.

18. Krajewska M, Krajewski S, Epstein JI, et al. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol, 1996; 148: 1567-76.

19. Augustin H, Hammerer PG, Graefen M, et al. Characterisation of biomolecular profiles in primary high-grade prostate cancer treated by radical prostatectomy. J Cancer Res Clin Oncol, 2003; 129: 662-8.

20. Pollack A, Cowen D, Troncoso P, et al. Molecular markers of outcome after radiotherapy in patients with prostate carcinoma: Ki-67, bcl-2, bax, and bcl-x. Cancer, 2003; 97: 1630-8.

21. McDonnell TJ, Troncoso P, Brisbay SM, et al. Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res, 1992; 52: 6940-4.

22. Viktorsson K, Lewensohn R, and Zhivotovsky B. Apoptotic pathways and therapy resistance in human malignancies. Adv Cancer Res, 2005; 94: 143-96.

23. Amirghofran Z, Monabati A, and Gholijani N. Apoptosis in prostate cancer: bax correlation with stage. Int J Urol, 2005; 12: 340-5.

24. Rubio J, Ramos D, Lopez-Guerrero JA, et al. Immunohistochemical expression of Ki-67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens. Eur Urol, 2005; 48: 745-51.

25. Royuela M, De Miguel MP, Bethencourt FR, et al. IL-2, its receptors, and bcl-2 and bax genes in normal, hyperplastic and carcinomatous human prostates: immunohistochemical comparative analysis. Growth Factors, 2000; 18: 135-46.

26. Chia SJ, Tang WY, Elnatan J, et al. Prostate tumours from an Asian population: examination of bax, bcl-2, p53 and ras and identification of bax as a prognostic marker. Br J Cancer, 2000; 83: 761-8.

27. Kim H, Rafiuddin-Shah M, Tu HC, et al. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol, 2006; 8: 1348-58.

28. Cartron PF, Gallenne T, Bougras G, et al. The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. Mol Cell, 2004; 16: 807-18.

29. Karst AM, Dai DL, Martinka M, and Li G. PUMA expression is significantly reduced in human cutaneous melanomas. Oncogene, 2005; 24: 1111-6.

30. Karst AM, Dai DL, Cheng JQ, and Li G. Role of p53 up-regulated modulator of apoptosis and phosphorylated Akt in melanoma cell growth, apoptosis, and patient survival. Cancer Res, 2006; 66: 9221-6.

31. Jansson A, Arbman G, and Sun XF. mRNA and protein expression of PUMA in sporadic colorectal cancer. Oncol Rep, 2004; 12: 1245-9.

32. Jansson AK, Emterling AM, Arbman G, and Sun XF. Noxa in colorectal cancer: a study on DNA, mRNA and protein expression. Oncogene, 2003; 22: 4675-8.

33. Therneau T AE An introduction to recursive partitioning using the rpart routine. Technical Report. Rocester: Section of Biostatistics, Mayo Clinic, 1997.

34. Breiman L FJ, Olshen RA, Stone CJ Classification and regression trees. California: CRC Press, 1984.

35. Fradet V, Lessard L, Begin LR, et al. Nuclear factor-kappaB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer. Clin Cancer Res, 2004; 10: 8460-4.

36. Koumakpayi IH, Diallo JS, Le Page C, et al. Low nuclear ErbB3 predicts biochemical recurrence in patients with prostate cancer. BJU Int, 2007; 100: 303-9.

37. Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, and Saad F. Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. Br J Cancer, 2006; 94: 1906-12.

38. Lessard L, Mes-Masson AM, Lamarre L, et al. NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int, 2003; 91: 417-20.

39. Koumakpayi IH, Diallo JS, Le Page C, et al. Expression and nuclear localization of ErbB3 in prostate cancer. Clin Cancer Res, 2006; 12: 2730-7.

40. Gannon PO, Alam Fahmy M, Begin LR, et al. Presence of prostate cancer metastasis correlates with lower lymph node reactivity. Prostate, 2006; 66: 1710-20.

41. Edwards J, Traynor P, Munro AF, et al. The role of HER1-HER4 and EGFRvIII in hormone-refractory prostate cancer. Clin Cancer Res, 2006; 12: 123-30.

42. Graf E, Schmoor C, Sauerbrei W, and Schumacher M. Assessment and comparison of prognostic classification schemes for survival data. Stat Med, 1999; 18: 2529-45.

43. Effron BT, RJ An introduction to the bootstrap. New York: Chapman and Hall, 1993.

44. Team RDC R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation fro Statistical Computing, 2006.

45. Hershko T and Ginsberg D. Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. J Biol Chem, 2004; 279: 8627-34.

46. Su X and Tsai CL. Tree-augmented Cox proportional hazards models. Biostatistics, 2005; 6: 486-99.

47. De Rose A and Pallara A. Survival trees: an alternative non-parametric multivariate technique for life history analysis. Eur J Popul, 1997; 13: 223-41.

48. LeBlanc M and Crowley J. Relative risk trees for censored survival data. Biometrics, 1992; 48: 411-25.

49. Glass TR, Tangen CM, Crawford ED, and Thompson I. Metastatic carcinoma of the prostate: identifying prognostic groups using recursive partitioning. J Urol, 2003; 169: 164-9.

50. Steuber T, Graefen M, Haese A, et al. Validation of a nomogram for prediction of side specific extracapsular extension at radical prostatectomy. J Urol, 2006; 175: 939-44; discussion 44.

51. Garzotto M, Park Y, Mongoue-Tchokote S, et al. Recursive partitioning for risk stratification in men undergoing repeat prostate biopsies. Cancer, 2005; 104: 1911-7.

52. Garzotto M, Beer TM, Hudson RG, et al. Improved detection of prostate cancer using classification and regression tree analysis. J Clin Oncol, 2005; 23: 4322-9.

53. Rubin MA, Bismar TA, Andren O, et al. Decreased alpha-methylacyl CoA racemase expression in localized prostate cancer is associated with an increased rate of biochemical recurrence and cancer-specific death. Cancer Epidemiol Biomarkers Prev, 2005; 14: 1424-32.

54. Altman DG and Royston P. What do we mean by validating a prognostic model? Stat Med, 2000; 19: 453-73.

55. Radespiel-Troger M, Hohenberger W, and Reingruber B. Improved prediction of recurrence after curative resection of colon carcinoma using tree-based risk stratification. Cancer, 2004; 100: 958-67.

56. Stapleton AM, Zbell P, Kattan MW, et al. Assessment of the biologic markers p53, Ki-67, and apoptotic index as predictive indicators of prostate carcinoma recurrence after surgery. Cancer, 1998; 82: 168-75.

57. Zeng L, Rowland RG, Lele SM, and Kyprianou N. Apoptosis incidence and protein expression of p53, TGF-beta receptor II, p27Kip1, and Smad4 in benign, premalignant, and malignant human prostate. Hum Pathol, 2004; 35: 290-7.

58. Keshgegian AA, Johnston E, and Cnaan A. Bcl-2 oncoprotein positivity and high MIB-1 (Ki-67) proliferative rate are independent predictive markers for recurrence in prostate carcinoma. Am J Clin Pathol, 1998; 110: 443-9.

59. Bubendorf L, Tapia C, Gasser TC, et al. Ki67 labeling index in core needle biopsies independently predicts tumor-specific survival in prostate cancer. Hum Pathol, 1998; 29: 949-54.

60. You H, Pellegrini M, Tsuchihara K, et al. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med, 2006; 203: 1657-63.

61. Obexer P, Geiger K, Ambros PF, Meister B, and Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. Cell Death Differ, 2007; 14: 534-47.

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Table 1. Patient cohort characteristics. Mean patient follow up was 72 months.

Overall patient cohort	
Age median (min-max)	
Cancer-free patients	35 (15-45)
PCa patients	62 (49-70)
AIPCa patients	79 (65-95)
Primary Prostate Cancer Patient Cohort	
Stage	
Stage 2	34
Stage 3	28
Invasion	
Extraprotatic extension	20
Lymph node imetastasis	9
Seminal vesicle invasion	9
Became Hormone-refractory	5
Had Prostatitis	1
Gleason Score	
Gleason 4	8
Gleason 5	14
Gleason 6	13
Gleason 7	17
Gleason 8-9	10
Pre-operative PSA	
<10 ng	32
>10 ng	28
Not available	2
PSA relapse	
Relapse	35
No relapse	27
Surgical margins	
Negative	31
Positive	31
Deaths within follow-up period	9

Table 2. Brier scores and associated 95% CI for selected RPART models. 95% CI were calculated from 200 bootstrapped samples.

Parameters included in the RPART model	Rank #	IBS	95 % CI
NOXA + PUMA + margin	1	0.107	0.065-0.128
NOXA + margin + PSA	6	0.114	0.069-0.142
Margin + Gleason	23	0.132	0.09-0.16
Margin + PSA	31	0.135	0.093-0.157
NOXA + PUMA	59	0.155	0.123-0.207

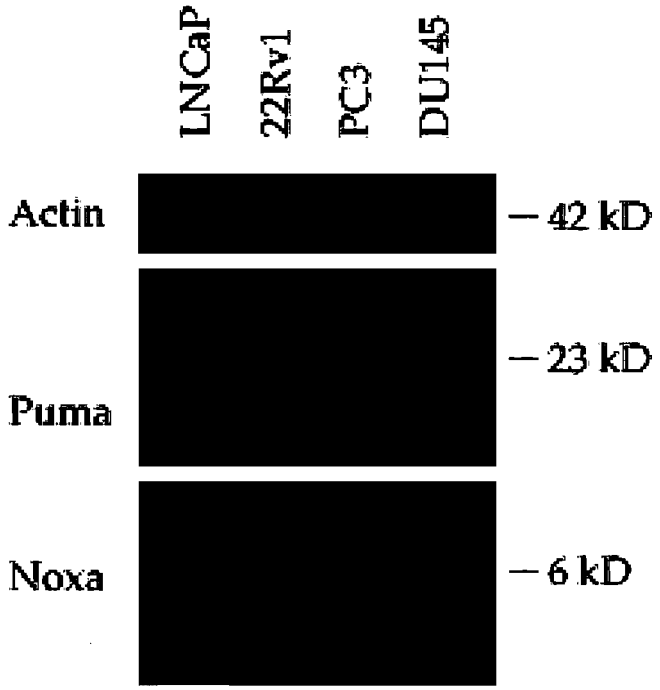


Figure 1 NOXA and PUMA expression in PCa cell lines

Figure 1. NOXA and PUMA expression in PCa cell lines. The Western blot was probed for NOXA, PUMA and actin in whole cell extracts obtained from androgen-responsive (LNCaP, 22Rv1) and androgen-independent PCa cell lines. Because the anti-NOXA and anti-PUMA antibodies used here recognized their respective targets with little background, they were subsequently deemed adequate for immunohistochemistry.

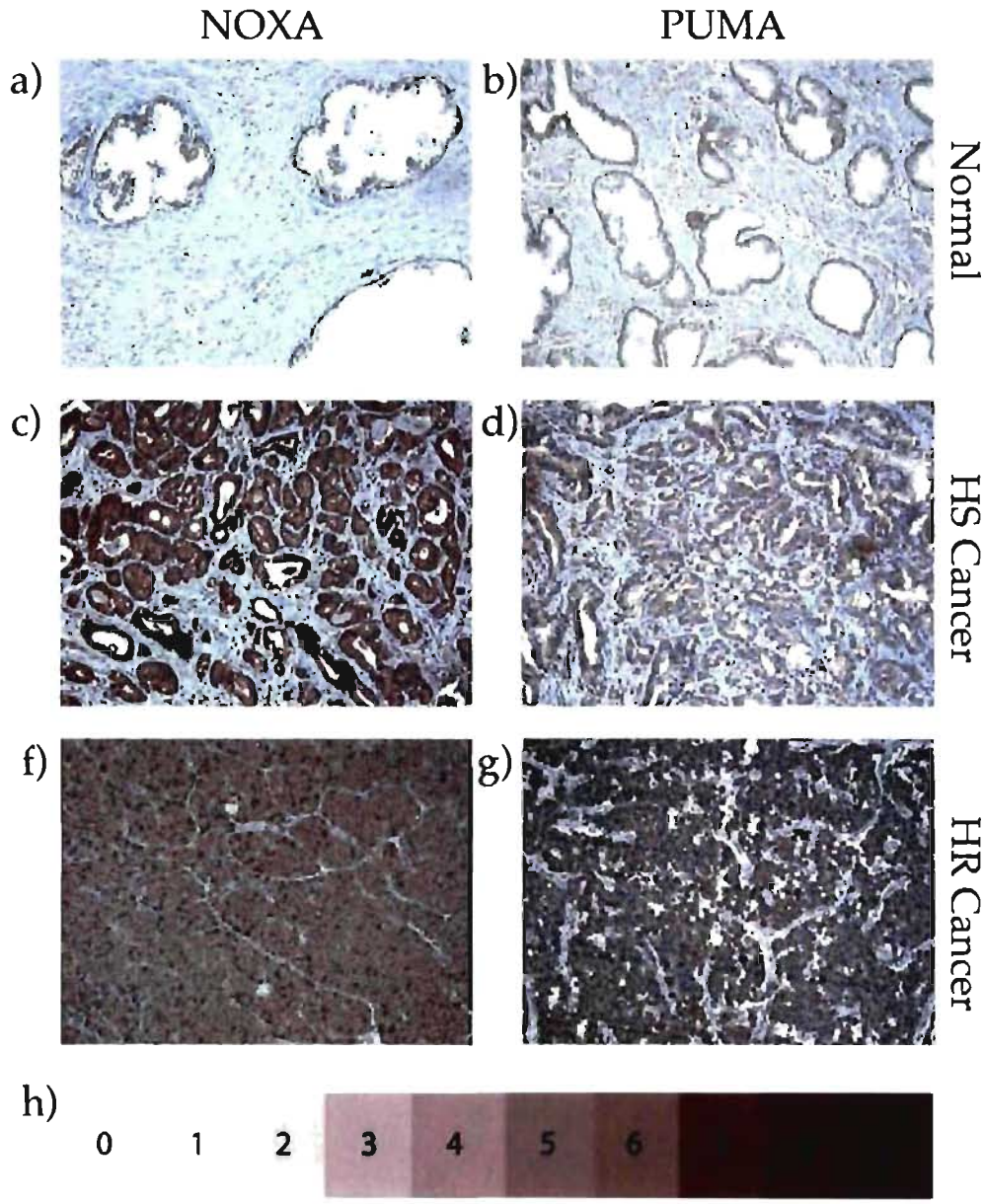
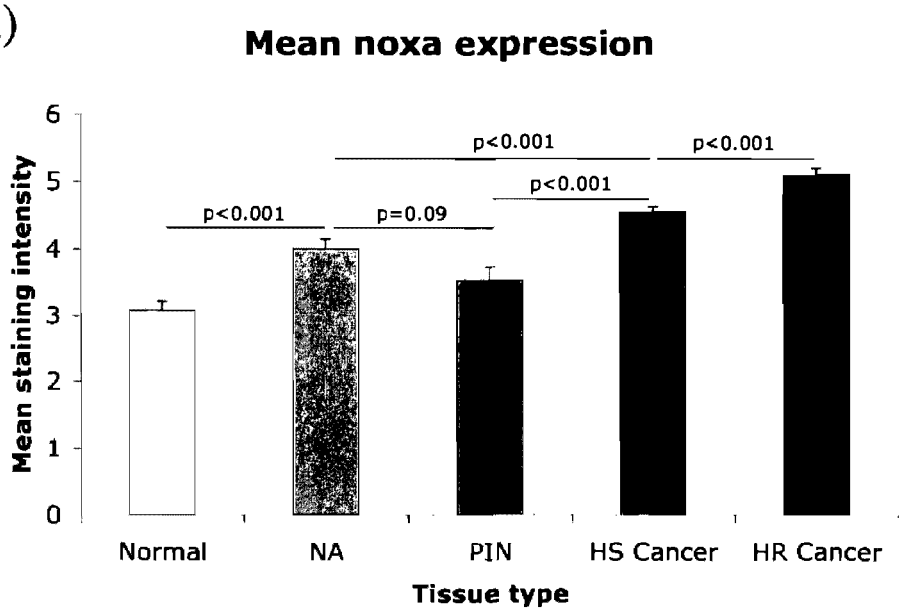


Figure 2. NOXA and PUMA expression in prostate tissues

Figure 2. Immunohistochemical staining of paraffin-embedded prostate tissues using anti-NOXA and anti-PUMA antibodies. A-B) Normal prostate tissue probed for NOXA and PUMA respectively. Note enhanced staining in the basal cell layer. C-D) Hormone-sensitive prostate carcinomas probed for NOXA and PUMA respectively. F-G) Hormone-refractory TURP specimens stained using anti-NOXA and anti-PUMA antibodies respectively. H) Color scale standard used for assessment of pictures obtained from tissue microarrays probed for NOXA and PUMA. Scale was constructed from several digital pictures evaluated in the study as described in materials and methods. Numeric values correspond to the associated intensity score.

a)



b)

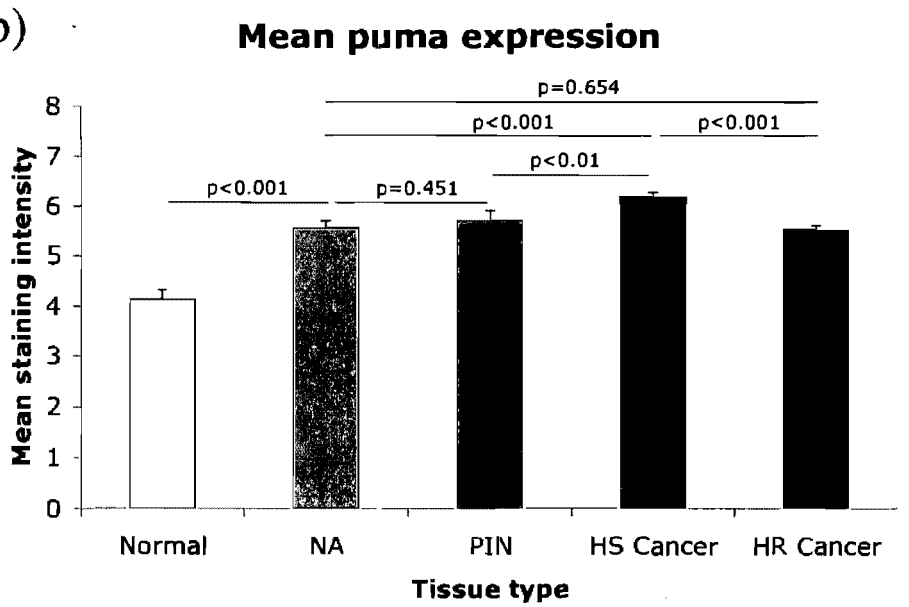


Figure 3. Average NOXA and PUMA expression in prostate tissue subtypes.

Figure 3. Average NOXA (A) and PUMA (B) expression in prostate tissue subtypes. Average was calculated over all the available cores in each subtype category. Normal = normal prostate tissue from autopsied patients (NOXA n=94; PUMA n=96 cores), NA= normal tissue found adjacent to cancer in radical prostatectomy specimens (n=91 cores), PIN = PIN tissue obtained from radical prostatectomy (n=43), HS cancer = hormone-sensitive cancer tissues obtained by radical prostatectomy (NOXA n=225; PUMA n=227), HR cancer = hormone-refractory cancer tissue obtained from TURP specimens (n=159). Two independent pathologists verified all core classifications. Error bars represent the standard error. Associated p-values were calculated using the Kruskal-Wallis non-parametric test. P-values under 0.05 were considered significant.

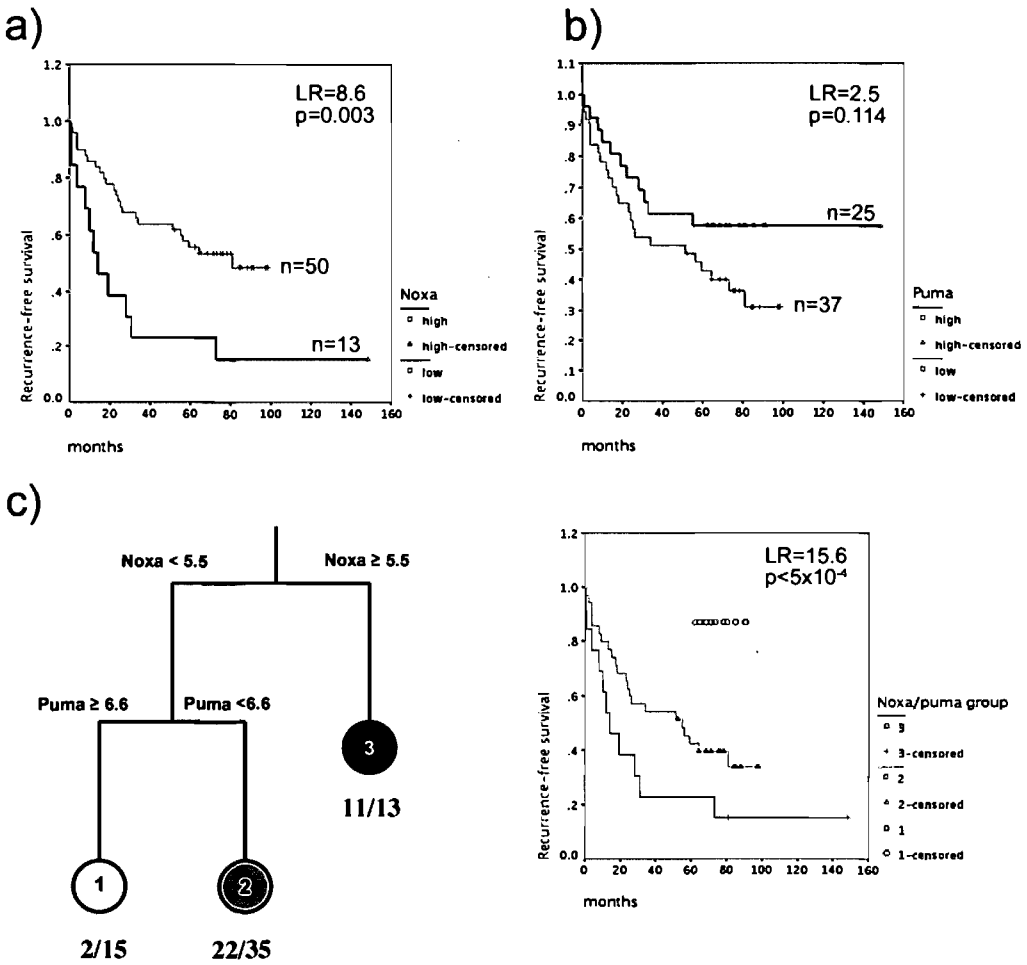


Figure 4. NOXA and PUMA expression in relation to the onset of biochemical recurrence following radical prostatectomy

Figure 4. Kaplan Meier plots for NOXA and PUMA categorized using optimal cutpoints obtained by RPART. A) NOXA expression in PCa is associated to BCR. Low (thin line) indicates average patient NOXA staining intensity was below 5.5 whereas High (thick line) indicates NOXA staining intensity over or equal to 5.5. B) PUMA expression alone does not significantly predict the onset of BCR. Low (thin line) indicates average patient PUMA staining intensity was below 6.6 whereas High (thick line) indicates PUMA staining intensity over or equal to 6.6. C) RPART model obtained for combined NOXA and PUMA. IBS=0.155 (95% CI=0.123-0.2073). Circled numbers correspond to the groups depicted in the associated Kaplan Meier plot shown in the right panel. Immediately below circled numbers, fractions (in bold font) represent number of patients that relapsed/number of patients in the group. LR=Log Rank; P= p-value. Note that p-value and Log Rank statistics should be considered as purely descriptive measures (see materials and methods).

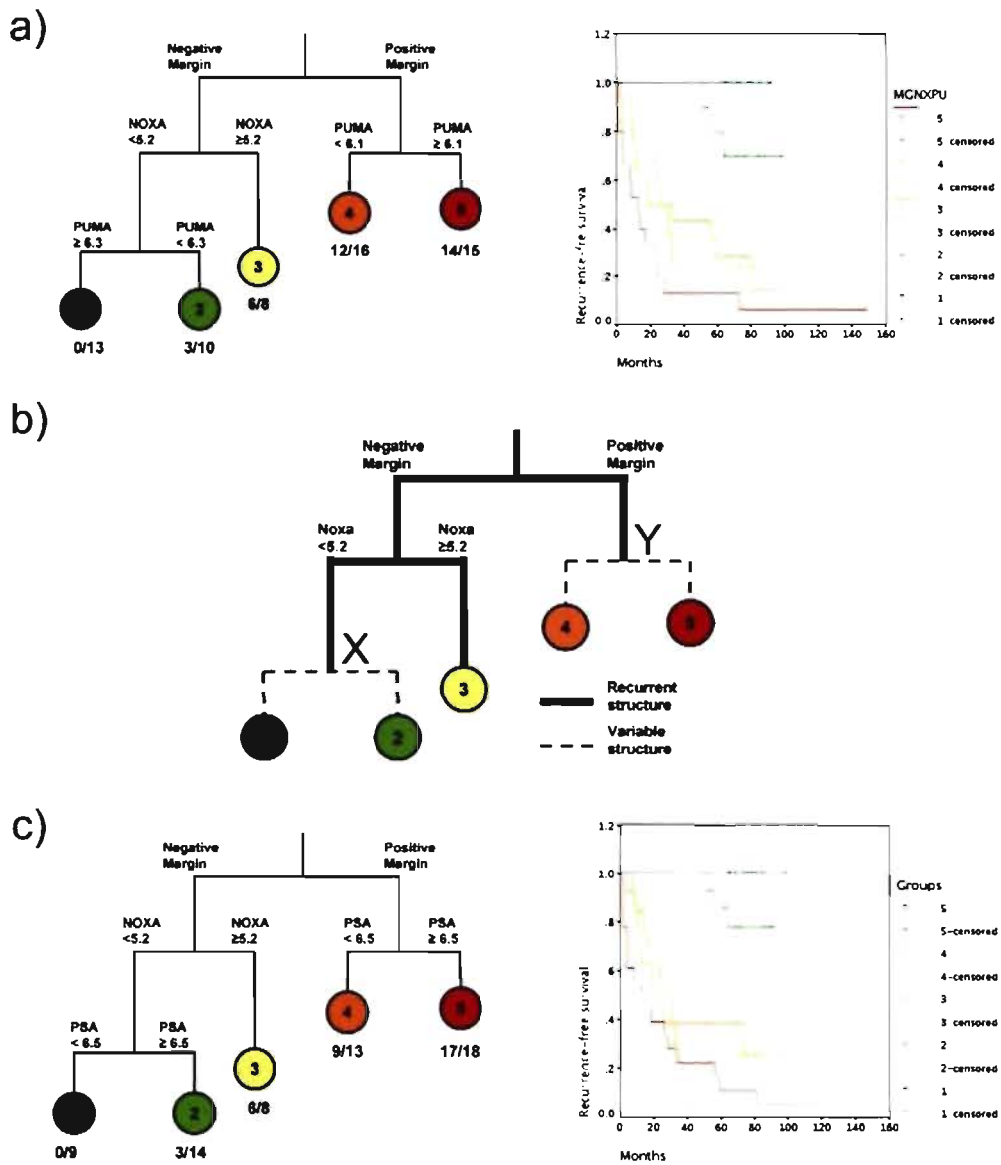


Figure 5. Top-ranking multivariate survival tree models.

Figure 5. Kaplan Meier plots for RPART model including NOXA, PUMA and clinical markers. A) Top ranking RPART model obtained in the study (MGNXPU). IBS=0.107 (95% CI=0.065-0.128). The left panel shows the associated survival tree where the numbers within circles corresponds to the MGNXPU groups depicted in the Kaplan-Meier plots (right panel). B) General survival tree structure determined from the top 9 models ranked by IBS. Recurrent structures (bold line) were those present in all of the top 9 ranking models. X and Y denote node-splitting variables (discontinuous line) within the top 9 models. In the top 9 ranking models, splitting parameter X was either PUMA, Gleason, pre-operative PSA. One model exhibited no variable for X (no split). Splitting variable Y was either PUMA, Gleason, pre-operative PSA, or NOXA. C) Favored RPART model obtained from the top nine RPART models from this study (Rank#6). IBS=0.113 (95% CI=0.069-0.142). Numbers within circles in the survival tree (left panel) correspond to the groups depicted in the Kaplan-Meier plots right panel. In A and C, the fractions below the colored circles represent the number of patients relapsed/number of patients in the group.

CHAPTER IV

Co-Assessment of Cytoplasmic and Nuclear Androgen Receptor Localization in Prostate Specimens: Potential Implications for Prostate Cancer Development and Prognosis

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Author contributions:

The writing of the manuscript and the majority of the work presented in his paper were done by the first author. All authors contributed to editing the paper. Abdulhadi Aldejmah helped with immunohistochemistry experiments and evaluation of the stained tissue arrays. Ali Filali Mouhim played an integral role in the survival tree analyses. Mona Alam Fahmy, Hervé Koumakpayi, Kaniska Sircar and Louis R. Bégin were pivotal in the construction of the tissue arrays.

Keywords: Androgen Receptor, Sub-cellular Localization, Biochemical Recurrence, Prostate Cancer, Survival Trees

Abstract

Background: The role of the androgen receptor (AR) in the development of androgen-independent (AI) prostate cancer (PCa) is well established. However, the contribution of nuclear AR in the early stages of PCa and its value as a marker for predicting biochemical recurrence (BCR) following radical prostatectomy (RP) remains controversial. The goal of this study was to address this issue by co-assessing cytoplasmic and nuclear AR expression in prostate tissues.

Materials and Methods: Archival prostate specimens from cancer-free patients (n=43), hormone-sensitive (HS) PCa patients (n=62), and AIPCa patients (n=30) were used to construct tissue microarrays (total n=135). Prostatic intra-epithelial neoplasia (PIN) as well as non-neoplasic tissues found adjacent to HSPCa (NA) were also included. Nuclear and cytoplasmic AR expression was scored by two observers using a composite scale following immunohistochemical detection of the AR. The nuclear/cytoplasmic AR expression ratio was also computed. Univariate Kaplan-Meier plots, as well as multivariate Cox and survival tree analyses were subsequently used to assess the ability of the AR to predict BCR in the sub-cohort of HSPCa patients.

Results: We found that the most striking increase in nuclear AR staining intensity when comparing normal prostate tissues from cancer-free patients to NA. Cytoplasmic AR expression was highest in AIPCa and increased considerably in relation to HSPCa. The nuclear/cytoplasmic AR expression ratio was highest in NA and PIN. In univariate analyses, we found that low nuclear AR, low cytoplasmic AR, and a high nuclear/cytoplasmic AR expression ratio were associated with BCR. Although cytoplasmic AR was an independent predictor of BCR in a Cox multivariate model (Hazard Ratio=2.736, 95% confidence interval 1.228-6.091, p-value=0.014), survival tree analyses suggest a complex relationship between AR expression and clinico-pathological features.

Conclusion: We propose that increased nuclear AR expression may be a precursor to PCa and that cytoplasmic AR could contribute to the AIPCa phenotype. We also conclude that the predictive ability of the AR may be closely linked to clinico-pathological features.

Introduction

Prostate cancer (PCa) remains a leading cause of cancer-related death in North-American men (1). Though localized forms of the disease can often be successfully treated by surgery or radiotherapy, a significant proportion of patients having undergone such interventions are at risk of disease relapse. Particularly, patients exhibiting an early rise in serum prostate specific antigen (PSA) levels (termed biochemical recurrence or BCR) following surgery are more likely to die from the disease (2). As such, it is likely that this specific group of patients would benefit from more aggressive treatment whereas in contrast, a less invasive approach may be more appropriate for patients at low risk of recurrence. Consequently, a growing number of studies have attempted to identify molecular markers that can accurately predict whether and how quickly patients will relapse following surgery.

Since the pioneering works of Huggins and Hodges(3), the mainstay for treatment of advanced or recurrent PCa has been androgen deprivation therapy. Though this treatment modality can increase patient life by several years, it eventually fails, giving rise to androgen-independent PCa (or AIPCa) for which treatment is essentially palliative (4-7). The principle target of androgen deprivation (or blockade) is the androgen receptor (AR), a nuclear receptor family transcription factor involved in prostate development, which is essential for growth and survival of the normal prostate epithelium. Bound by androgens, this transcription factor is released from its sequestration by heat shock proteins and translocates to the nucleus where it drives the expression of various genes, many of which are involved in cell proliferation and survival (reviewed in (8)). This has led to the suggestion that various AR-dependent mechanisms can drive the progression towards AIPCa. These include AR mutations within various domains involved in transactivation and ligand binding, AR gene amplification and/or up-regulation, as well as increased expression of AR co-activators or hyper-activation of pathways leading to increased AR transactivation (reviewed in (9)).

Although much evidence supports a role for the AR, either direct or indirect, in the development of AIPCa following androgen depletion therapy, the

link between the AR and PCa aggressiveness prior to treatment by androgen deprivation is far from clear. Indeed, the literature is divided as to whether expression of the AR is associated to more or less aggressive disease. Some studies have reported decreased expression of the AR in cancerous prostate tissue as opposed to benign prostate tissue (10, 11). However, this is not consistently found (9). Similarly, there is no consensus as to how the AR relates to disease relapse following surgery (10, 12-15). It is likely that these conflicting findings are largely responsible for the absence of the AR from the relatively short list of clinically used prognostic markers for PCa.

Likely due to the well established role of the AR as a transcription factor, most of the immunohistochemical studies that have looked at the AR in prostate tissues obtained from patients have either not discriminated between nuclear and cytoplasmic staining (10, 12-14) or have focused exclusively on AR nuclear localization (15, 16). Nonetheless, several AR binding partners have been identified and while some are nuclear proteins (e.g CBP/p300 and SRC-1 (17)), others reside in the plasma membrane or cytoplasm (e.g EGFR (18), Caveolin-1 (19)) or can shuttle between nuclear and cytoplasmic compartments (e.g cyclin D1 (20), Rb (21), Akt (22), NF-κB (23)). The AR has even been reported to interact with mitochondrial proteins (COXVb (24)). Altogether, this suggests that the expression of the AR in the cytoplasm may also have an impact on PCa progression.

In light of this, we set out to study cytoplasmic AR expression in parallel with nuclear AR expression in prostate tissues of various sources. We looked at the intensity of both cytoplasmic and nuclear AR staining using tissue micro-arrays containing normal prostate tissue, primary PCa and its adjacent non-neoplasic tissue, as well as androgen-independent specimens, representing a total of 135 patients. We then evaluated the ability of nuclear and cytoplasmic AR to predict BCR within patients that had undergone radical prostatectomy, but that were not subjected to androgen deprivation therapy prior to relapse. To evaluate the prognostic ability of the AR in combination with established clinical markers, we used Cox proportional hazard models as well as survival trees, a method based

on recursive partitioning of the covariate space whose graphical output facilitates the visualization of prognostic groups reflecting multi-marker interactions.

Materials and Methods

Patient cohort

A total of 51 normal prostate specimens were obtained from cancer-free patients. An additional 64 paraffin-embedded human primary PCa specimens from patients who had undergone radical prostatectomy between 1993 and 2000 were also used. Futhermore, trans-urethral resections of the prostate (TURP) specimens from 30 AIPCa patients were obtained. Regions of non-neoplasic and cancerous epithelial tissue were identified by two pathologists and subsequently spotted on tissue micro-arrays. In the sub-cohort of 64 primary PCa tumors, which was used for retrospective prognostic studies, no patient received preoperative hormone therapy and all cases had a clinical follow-up of at least 5 years or until death (average follow up of 72 months). No age difference was observed between the group of patients who relapsed and the group that did not. Postoperative PSA was available for all patients. The time to biochemical recurrence (BCR) was defined as the time elapsed between the date of surgery and the date where PSA first increased from undetectable levels to above 0.3 ng/ml and rising, consistent with previous publications (25-27). Non-relapsed patients had a PSA remaining below 0.3 ng/ml after radical prostatectomy. For PCa specimens, the final pathological staging, grading and histo-pathological diagnosis was based on the pathology report in agreement with the review from an independent pathologist. Specimens were obtained from consenting patients and the institutional ethics review committee approved the study.

Tissue array construction and verification

Tissue arrays containing a total of 613 one millimeter-wide cores of prostate tissues were built and used for IHC studies. For the sub-cohort containing normal tissue cores obtained from 51 autopsied patients, two cores per patient were spotted on a tissue micro-array. For the prognostic sub-cohort of primary

tumors, two non-neoplasic and four cancerous cores per patient were spotted on tissue arrays. For the hormone-refractory TURP sub-cohort, four cores from each patient were included on a tissue-microarray. Following tissue-array construction, 4 μm thick cross-sections were put on glass slides and stained with hematoxylin/eosin as well as for cytokeratin 34βE12 and reviewed by two pathologists. All cores were subsequently re-categorized as containing no epithelial cells, non-neoplasic epithelium, focal atrophy, PIN or adenocarcinoma. Cores containing no epithelial cells or focal atrophy were not considered in the analysis. Following reclassification, the final specimen cohort consisted of 601 cores representing 43 patients with normal prostate tissues, 62 patients presenting primary PCa tissues and 30 patients with hormone-refractory-PCa for a total of 135 patients. Patient characteristics are summarized in Table 1.

Immunohistochemistry

Samples were immunostained with an anti-AR antibody (NeoMarkers, Clone AR-441, MS-443-P) diluted 1:250 in PBS. Primary antibody detection was done using the LSAB 2 peroxidase system from Dako Diagnostics Inc (CA, USA). Staining was performed as described previously (25, 28-30). Briefly, tissue samples were deparaffinized, rehydrated, and treated with 0.3% H_2O_2 to eliminate endogenous peroxidase activity. An antigen retrieval step was performed using 10 mM citrate buffer (pH 6.0) applied for 17.5 minutes at 95°C. All following steps were performed at room temperature. The sections were blocked with a protein-blocking serum-free reagent (Dako) and incubated with primary antibody for 60 minutes, followed by a 20 minute treatment with the secondary biotinylated antibody (Dako), washed 5 minutes in PBS, and then incubated for 20 minutes with streptavidin-peroxidase (Dako). Following an additional 5 minute PBS wash, reaction products were developed with diaminobenzidine (Dako) containing 0.3% H_2O_2 as a substrate for peroxidase. Nuclei were counterstained with Harris hematoxylin (Sigma-Aldrich, MO, USA). No non-specific secondary antibody staining was observed when PBS was used instead of the primary antibody.

Scoring procedure

Digital images were obtained of each tissue micro-array core using an Olympus BX51 microscope equipped with the Q capture imaging software (Olympus, London, UK). Two observers quantified average nuclear and cytoplasmic epithelial cell AR staining intensity using a color scale (0-9, Fig.1d) constructed from the various staining intensities observable in the digital images using the eyedropper tool in Adobe Photoshop 7.0 as described previously (31).

Statistics

Mean cytoplasmic and nuclear staining intensities of epithelial cells associated with either normal tissue, non-neoplasic tissue adjacent to cancer (NA), PIN, HSPCa and AIPCa tissues were calculated. The nuclear/cytoplasmic AR ratio was also calculated for each core and averaged per tissue sub-type. Notably, all cores exhibited cytoplasmic staining over zero and all nuclear/cytoplasmic intensity ratios were computable. Kruskal-Wallis non-parametric tests were used to assess statistical significance of observed differences in mean staining intensity between tissue sub-types. All correlation coefficients were computed using Spearman's non-parametric test. Cutoff determination and survival tree construction was done using the RPART (Recursive PARTitioning) libraries (32), which extends the CART (Classification and Regression Trees) routine (33). Model accuracy was assessed using the integrated Brier score for censored data (IBS) (34. We used 200 bootstrap {Effron, 1993 #35) samples to compute the 95% confidence interval (CI) on the IBS. Survival tree growth was controlled using the minimum splitting (*minsplit*) criterion implemented in RPART. This parameter controls the minimum number of observations that must exist in a node, in order for a split to be attempted. For combination models including cytoplasmic AR intensity and/or nuclear AR intensity or the ratio of both with the four clinical parameters (stage, surgical margin status, pre-operative PSA and Gleason score), RPART models were generated using three different values of *minsplit* (20, 25 and 30). All the generated combination models were ranked according to IBS. Optimal cutoffs for cytoplasmic AR, nuclear AR, and nuclear /

cytoplasmic AR ratio were defined as values associated to the root node of the survival trees generated from the variables taken individually (*minsplit*=25). Kruskal-Wallis, Spearman, and Kaplan-Meier analyses were performed using Statistical Package for the Social Sciences (SPSS) version 11 (SPSS, Inc., Chicago, USA). Tree building and the calculation of IBS were carried out in the R statistical package (version 2.4.0; (35)), using rpart and ipred packages respectively (www.cran.r-project.org).

Results

Cytoplasmic and Nuclear Androgen Receptor Expression in Prostate tissues

To assess AR expression and subcellular localization at various steps of PCa progression, we used tissue microarrays containing prostate tissues originating from autopsied patients presenting no signs of PCa (n=43), from radical prostatectomies (RP) of patients not subjected to prior hormone therapy (n=62), and from TURP specimens of AIPCa patients (n=30). In addition, regions of normal tissue, PIN, and cancer were identified within RP specimens and included in the tissue microarrays. An antibody previously shown to be specific for the AR was used to stain the tissue microarrays (36, 37). This antibody stained both the cytoplasm and the nucleus of prostate epithelial cells to various extents as expected (Fig.1a-c). Average cytoplasmic and nuclear AR staining intensity in cores from each tissue type was assessed from digital images using a color scale (Fig. 1d) as described (see materials and methods).

Looking at average cytoplasmic AR staining intensity (Fig. 2a), we found a minimal but significant increase in non-neoplasic tissues adjacent to cancer (NA) as opposed to normal tissues obtained from cancer-free patients ($p<0.0001$). A slight decrease in cytoplasmic AR was observed in PIN tissues as compared to NA tissues ($p=0.0145$), although cytoplasmic AR in PIN was not significantly different compared to normal tissues obtained from autopsied patients. In contrast, there was a significant increase in cytoplasmic AR within hormone-sensitive cancer tissues (obtained by RP) as compared to both normal and NA tissues ($p<0.0001$). Notably, the highest expression of cytoplasmic AR was observed in

AIPCa reaching substantially higher levels than those observed in hormone-sensitive (HS) PCa (4.9 vs 2.8 respectively, $p<0.0001$).

In contrast with what was observed for cytoplasmic AR, we detected the most striking increase in nuclear AR staining intensity when comparing NA tissues to normal tissues obtained from cancer-free patients (Fig 2b). Although there was a slight increase in nuclear AR in HSPCa as compared to NA tissues ($p=0.002$), we observed a modest but significant decrease in nuclear AR in AIPCa as compared to HSPCa ($p=0.015$). We then explored how the ratio of nuclear / cytoplasmic AR staining intensity varied in PCa progression (Fig. 2c). We found that this ratio increased substantially from normal to NA tissues ($p<0.0001$). From a maximum value in PIN tissues, nuclear/ cytoplasmic ratio decreased slightly but significantly in HSPCa. From HSPCa to AIPCa, there was a substantial drop in the nuclear / cytoplasmic AR ratio ($p<0.0001$), which returned to values close to those observed in normal tissues obtained from cancer-free patients.

Cytoplasmic and nuclear AR staining intensities are associated to biochemical recurrence.

To determine whether cytoplasmic and/or nuclear AR staining intensity could predict BCR following surgery, we focused on the sub-cohort of 62 HSPCa patients that did not undergo androgen ablation prior to RP or prior to BCR. We re-categorized cytoplasmic, nuclear and nuclear/cytoplasmic AR values according to an optimal cutoff (see material and methods). Survival curves were subsequently plotted using the categorized data. For cytoplasmic AR, we found that low staining intensity (<2.1) was associated with an increased risk of BCR (Fig. 3a, Log rank or LR=10.82, $p=0.001$). Similar results were obtained for nuclear AR staining intensity (Fig. 3b, low < 7.3, LR=9.018, $p=0.008$). In contrast, a high nuclear/cytoplasmic ratio (≥ 3.5) was associated with an increased risk of BCR (Fig. 3c, LR=9.25, $p=0.0023$). As optimal cutoffs were obtained from the same cohort, p-values associated to the Log Rank statistic from Kaplan Meier plots are subject to over-fitting and are best interpreted qualitatively.

Cytoplasmic and nuclear AR staining intensity: relationship with clinical markers and patient outcome.

We next determined whether the association between cytoplasmic and nuclear AR expression with BCR was due their potential correlation with clinico-pathological parameters. We found that cancer-positive surgical margins were associated with decreasing cytoplasmic AR, decreasing nuclear AR, as well as increasing nuclear/cytoplasmic AR ratio, when these were assessed as continuous variables (Table 2). When categorized according to optimal cutoffs, we found that low cytoplasmic AR expression as well as high nuclear/cytoplasmic AR ratio also correlated with increasing pathological stage, extracapsular invasion and lymph node invasion (Table 2). In light of this, we assessed whether AR expression in the cytoplasm, nucleus, or both (ratio), could be useful to predict BCR in combination with tumor stage, Gleason score, surgical margin status and pre-operative PSA. Using Cox multivariate proportional hazard models on the sub-cohort of 62 patients, we found that un-categorized AR staining intensity was not an independent predictor of BCR, irrespectively of the sub-cellular compartment assessed (data not shown). However, when categorized according to the optimal cutoff, cytoplasmic AR expression was retained in a multivariate model that also contained tumor stage and Gleason score (Hazard Ratio=2.736, 95% confidence interval 1.228-6.091, p-value=0.014), where patients exhibiting low cytoplasmic AR expression were at higher risk of BCR.

To gain a more visual perspective on the relationship between AR expression, clinico-pathological parameters, and BCR, we proceeded to generate RPART survival trees using cytoplasmic and nuclear AR as well as the nuclear/cytoplasmic AR ratio, in combination with pathological stage, surgical margin status, Gleason score and pre-operative PSA (see materials and methods, (31)). These models were then classified according to the IBS, a measure of the predictive accuracy where a lower value indicates better outcome prediction. The top ranking survival tree (IBS=0.118, 95% confidence interval=0.081-0.144) included surgical margin status, cytoplasmic AR expression, and Gleason score (Fig. 4). Notably, the top 20 trees contained cytoplasmic and/or nuclear AR or the

nuclear/cytoplasmic AR ratio. However, there was no consistently re-occurring tree structure in the top ranking models (data not shown). As a reference, the top-ranking model composed exclusively of clinical markers (rank # 21 of 82 overall) was one that contained surgical margin status and Gleason score (IBS=0.132, 95% confidence interval = 0.096 – 0.165, not shown) as was found in a previous study (31).

Surprisingly, in the top ranking survival tree model, low cytoplasmic AR (<3.4) was associated with better prognosis (Fig. 4a), specifically within the subset of patients with negative surgical margins. We thus looked at whether cytoplasmic, nuclear or nuclear/cytoplasmic AR ratio predicted different outcomes depending on the subgroup of patients delineated by clinical parameters within the survival trees. For cytoplasmic AR, we found that while a low expression was commonly associated with better prognosis in patients with cancer-negative surgical margins, this was instead associated with worse prognosis in patients exhibiting high stage tumors. In contrast, high nuclear AR was generally associated with good prognosis within patients exhibiting low stage tumors or negative surgical margins. In concordance with both of these findings, low stage tumors with a high nuclear/cytoplasmic AR ratio indicated a better prognosis as exemplified in the survival tree that ranked second overall (IBS=1.22, 95% CI=0.08-0.14, Fig 4b).

Discussion

In this study we found clear differences between the expression of the AR in cytoplasmic and nuclear compartments within histo-pathological subtypes of prostate tissues (Fig 1a-c, 2a-c). Notably, we found that while cytoplasmic AR expression only slightly increased in NA tissues as opposed to normal tissues obtained from cancer-free patients (Fig. 2a), nuclear AR staining intensity was in comparison substantially higher in NA and PIN tissues (Fig. 2b). One possible explanation why AR nuclear localization is more prevalent in NA and PIN tissues as compared to normal tissues from cancer-free patients is the difference in average patient age (35 in cancer-free patients vs. 62 in HSPCa patients, Table 1).

Notably, AR expression has been observed to increase with age in a rat model, an event that was linked to spontaneous prostate hyperplasia (38). On the other hand, we did not find any correlation between patient age and AR expression within HSPCa patients (Table 2). Hence, another possibility is that AR expression is up regulated in response to one or more genetic events. Interestingly, increased levels of AR protein are observed in NKX3.1 knockout mice, where NKX3.1 is thought to act as a negative regulator of AR expression (39). Notably, the loss of NKX3.1 has been suggested to be an early event in the development of localized PCa (40).

Although further studies will be necessary to address the cause, our data suggest that increasing AR nuclear localization could be a precursor to PCa development and that additional genetic events may be important for PCa progression. Consistent with this notion, it has been previously shown that prostate-specific AR over-expression in transgenic mice leads to enhanced cell proliferation and PIN but does not lead to adenocarcinoma (41). In this context, it is tempting to speculate that increased AR expression could act as a "primer" for further transformation of prostate cells. This idea is particularly appealing in light of the recent discovery of chromosomic fusions between oncogenic ETS family transcription factors and the AR-regulated TMPRSS2 gene, which are detectable in over 80% of primary PCa tumors (42, 43).

On the other hand, we found that although AR staining intensity in both nuclear and cytoplasmic compartments was significantly increased in HSPCa as compared to NA tissue, this difference was much less impressive than when comparing normal tissues to NA tissues, particularly at the nuclear level (Fig 2a-b). Furthermore, we observed a slightly lower nuclear AR staining in AIPCa tissues as opposed to HSPCa (Fig. 2b). Although these findings support an early rather than late contribution of the AR in PCa progression, they do not necessarily absolve the AR as a major contributor in the later stages of PCa. Of note, several co-activators can enhance AR transcriptional activity. Hence, it is possible that AR co-activators can be up regulated in parallel, as has been observed for CDC25B when comparing PCa tumors to their adjacent normal tissues (44). Similarly, we cannot exclude the possibility that at least in some cases, the AR

may harbor mutations that lead to constitutive transcriptional activation of the AR (45). In this scenario, one could expect that AR transcriptional activity may be heightened not only in HSPCa, but also in AIPCa, even though nuclear levels are only modestly increased or similar to those observed in NA tissues.

In addition to looking at AR staining in the cytoplasm and nucleus of prostate epithelial cells, we deemed it relevant to also compute the nuclear/cytoplasmic AR staining intensity ratio. This was done for two main reasons. First, we rationalized that an increasing nuclear / cytoplasmic ratio should theoretically correlate with increasing AR transcriptional activity. Second, in the likelihood that certain tissues may exhibit differential staining solely on the basis of differences in tissue processing (as opposed to truly biologically significant differences), the nuclear / cytoplasmic ratio should remain the same and consequently act as an internal normalization control. Interestingly, the nuclear / cytoplasmic ratio revealed a clear propensity towards nuclear accumulation in NA and PIN tissues as compared to normal tissue from cancer-free patients. However, this ratio decreased in HSPCa and even further in AIPCa, reaching levels comparable to those observed in normal tissues (Fig. 2c).

Although we would expect that androgen ablation should lead to increased cytoplasmic AR and decreased nuclear AR, which is what we observe here (Fig 2a-c), it is clear that cytoplasmic AR fluctuates much more than nuclear AR in response to androgen deprivation (compare Fig 2a to 2b). However, overall AR expression in both compartments combined was far greater in AIPCa supporting results obtained in other studies looking at AR gene amplification and over–expression in AIPCa (reviewed in (8, 9, 46)). Importantly, although cytoplasmic localization of the AR may be an effect of androgen deprivation, it is a likely possibility that the striking increase in cytoplasmic AR could have repercussions on the AIPCa phenotype. Indeed, numerous potential AR binding partners that either mainly reside in the cytoplasmic compartment or that shuttle between the nucleus and cytoplasm have also been implicated in PCa progression including EGF receptors (14, 26, 29), Akt (27) and NF-κB (25, 47).

In the sub-cohort of patients having undergone RP that did not receive

hormone therapy prior to disease recurrence, we found that low AR expression in both cytoplasmic and nuclear compartments was associated to an increased risk of BCR (Fig. 3a-b). Interestingly, a high nuclear/cytoplasmic ratio was associated to a worse prognosis (Fig. 3c). This finding is particularly interesting because it may partly explain why the literature is so divided on the relationship between AR expression and both BCR and PCa progression. Notably, while our observation that low nuclear AR is associated to BCR is in line with one study (14), the nuclear/cytoplasmic AR ratio is rather in agreement with another study (10). Interestingly, the latter study also reported decreased nuclear AR expression in PCa as opposed to non-neoplasic tissues, similarly to what was observed here for the nuclear / cytoplasmic ratio (Fig. 2c). At the same time, other studies have also found increased nuclear AR in PCa (9), which is in line with our results looking exclusively at nuclear AR (Fig. 2b). In the framework where nuclear/cytoplasmic AR ratio is an internally normalized value, this suggests that certain conflicting results presented in the literature may be partly due to differences in tissue fixation between samples within each given study. Hence, it is likely that these discrepancies could be rectified by some means of data normalization using an internal control. As such, it would be interesting to look at the nuclear / cytoplasmic AR ratio in future immunohistochemistry, particularly those aiming to evaluate the AR as a prognostic marker.

Within the HSPCa patient cohort, we found that nuclear, cytoplasmic, and nuclear/cytoplasmic AR expression correlated with pathological stage and surgical margin status (Table 2). In addition, cytoplasmic and nuclear/cytoplasmic ratios also correlated with lymph node invasion and extracapsular invasion. Interestingly, we found that only cytoplasmic AR was an independent predictor of BCR in a multivariate Cox model. Using RPART to generate survival trees, we found that the top ranking survival tree model (lowest IBS) stratified BCR risk according to surgical margin status, cytoplasmic AR, and Gleason score. In this model, low cytoplasmic AR was associated with better outcome within patients with negative surgical margins (Fig 4a). Similarly, in the second highest ranked survival tree model, high nuclear/cytoplasmic ratio was associated with better

outcome in patients with low stage tumors (Fig. 4b).

When we further explored the interactions between cytoplasmic, nuclear, nuclear / cytoplasmic ratio and clinical parameters using all the generated survival trees, we found that the predictive value of AR expression was highly dependent on what clinical parameters were included in the model. Interestingly the prognosis associated to AR status was often counter to what was observed in the overall cohort (Fig. 3a-c) in patients with cancer-negative surgical margins (see Fig.4a). Perhaps this finding can be linked to an inherent difference between recurrent margin-negative and margin positive tumors, in relation to the type of PCa cells that remain following RP. For example, in order to be detected in histopathological cross sections, PCa cells remaining at and beyond the surgical margins may require a certain clustering density. In addition to the observation that positive surgical margin status correlates with both extra-prostatic (Spearman coefficient or SC = 0.621, $p<0.001$, data not shown) and seminal vesicle invasion (SC = 0.289, $p<0.05$; data not shown), this might suggest that in general, recurring tumors from positive margins initiate from denser, pre-established groups of cells than those remaining from tumors with "negative" surgical margins. In contrast, undetected PCa cells remaining from an excised tumor deemed to have cancer-free surgical margins are likely to be more scattered and detached. In this context, low cytoplasmic AR and/or high nuclear AR expression may more severely impact the survival or proliferation of these "scattered" cells, leading to a better outcome from the perspective of the patient. Notably, several groups have reported that exogenous expression of the AR in AR-negative PC3 cells reduces proliferation and induces apoptosis in the presence of androgens (48, 49). Although the biological significance of these findings will certainly require further investigation, our results suggest at the very least that AR expression may be intimately linked to clinico-pathological characteristics further implying that the composition of patient cohorts can have a strong influence on the conclusions drawn regarding the association between AR expression and the onset of BCR.

Conclusion

We propose that increased nuclear AR expression may be a precursor to PCa development and that other genetic events are likely required to initiate PCa transformation. We also conclude that the most significant change in AR expression in AIPCa occurs at the cytoplasmic level and propose that this may contribute to the AIPCa phenotype. We find that although low nuclear and low cytoplasmic expression is associated with PCa relapse following surgery, a high nuclear/cytoplasmic AR ratio is associated with an increased risk of early BCR. Overall, we conclude that although cytoplasmic AR is an independent predictor of BCR in a Cox model, extensive survival tree analyses suggest a complex interaction between AR expression and pathological features, which will require further evaluation in a larger cohort.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin. 2007 Jan-Feb;57(1):43-66.
2. Freedland SJ, Humphreys EB, Mangold LA, Eisenberger M, Partin AW. Time to prostate specific antigen recurrence after radical prostatectomy and risk of prostate cancer specific mortality. J Urol. 2006 Oct;176(4 Pt 1):1404-8.
3. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. CA Cancer J Clin. 1972 Jul-Aug;22(4):232-40.
4. Denis L, Murphy GP. Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. Cancer. 1993 Dec 15;72(12 Suppl):3888-95.
5. Culine S, Droz JP. Chemotherapy in advanced androgen-independent prostate cancer 1990-1999: a decade of progress? Ann Oncol. 2000 Dec;11(12):1523-30.
6. Crawford ED, Eisenberger MA, McLeod DG, Spaulding JT, Benson R, Dorr FA, et al. A controlled trial of leuprolide with and without flutamide in prostatic carcinoma. N Engl J Med. 1989 Aug 17;321(7):419-24.
7. Morris MJ, Scher HI. Novel strategies and therapeutics for the treatment of prostate carcinoma. Cancer. 2000 Sep 15;89(6):1329-48.
8. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer. 2001 Oct;1(1):34-45.
9. Buchanan G, Irvine RA, Coetzee GA, Tilley WD. Contribution of the androgen receptor to prostate cancer predisposition and progression. Cancer Metastasis Rev. 2001;20(3-4):207-23.
10. Li R, Wheeler T, Dai H, Frolov A, Thompson T, Ayala G. High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate: cancer

patients treated with radical prostatectomy. Am J Surg Pathol. 2004 Jul;28(7):928-34.

11. Olapade-Olaopa EO, Muronda CA, MacKay EH, Danso AP, Sandhu DP, Terry TR, et al. Androgen receptor protein expression in prostatic tissues in Black and Caucasian men. Prostate. 2004 Jun 1;59(4):460-8.
12. Amirghofran Z, Monabati A, Gholijani N. Androgen receptor expression in relation to apoptosis and the expression of cell cycle related proteins in prostate cancer. Pathol Oncol Res. 2004;10(1):37-41.
13. Dunsmuir WD, Gillett CE, Meyer LC, Young MP, Corbishley C, Eeles RA, et al. Molecular markers for predicting prostate cancer stage and survival. BJU Int. 2000 Nov;86(7):869-78.
14. Schafer W, Funke PJ, Kunde D, Rausch U, Wennemuth G, Stutzer H. Intensity of androgen and epidermal growth factor receptor immunoreactivity in samples of radical prostatectomy as prognostic indicator: correlation with clinical data of long-term observations. J Urol. 2006 Aug;176(2):532-7.
15. Theodoropoulos VE, Tsigka A, Mihalopoulou A, Tsoukala V, Lazaris AC, Patsouris E, et al. Evaluation of neuroendocrine staining and androgen receptor expression in incidental prostatic adenocarcinoma: prognostic implications. Urology. 2005 Oct;66(4):897-902.
16. Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, et al. Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer Res. 2001 Jan 15;61(2):423-7.
17. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. Mol Cell. 2002 Mar;9(3):601-10.
18. Bonaccorsi L, Muratori M, Carloni V, Marchiani S, Formigli L, Forti G, et al. The androgen receptor associates with the epidermal growth factor receptor in androgen-sensitive prostate cancer cells. Steroids. 2004 Aug;69(8-9):549-52.

19. Lu ML, Schneider MC, Zheng Y, Zhang X, Richie JP. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. J Biol Chem. 2001 Apr 20;276(16):13442-51.

20. Burd CJ, Petre CE, Moghadam H, Wilson EM, Knudsen KE. Cyclin D1 binding to the androgen receptor (AR) NH2-terminal domain inhibits activation function 2 association and reveals dual roles for AR corepression. Mol Endocrinol. 2005 Mar;19(3):607-20.

21. Yeh S, Miyamoto H, Nishimura K, Kang H, Ludlow J, Hsiao P, et al. Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. Biochem Biophys Res Commun. 1998 Jul 20;248(2):361-7.

22. Lin HK, Yeh S, Kang HY, Chang C. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A. 2001 Jun 19;98(13):7200-5.

23. Palvimo JJ, Reinikainen P, Ikonen T, Kallio PJ, Moilanen A, Janne OA. Mutual transcriptional interference between RelA and androgen receptor. J Biol Chem. 1996 Sep 27;271(39):24151-6.

24. Beauchemin AM, Gottlieb B, Beitel LK, Elhaji YA, Pinsky L, Trifiro MA. Cytochrome c oxidase subunit Vb interacts with human androgen receptor: a potential mechanism for neurotoxicity in spinobulbar muscular atrophy. Brain Res Bull. 2001 Oct-Nov 1;56(3-4):285-97.

25. Fradet V, Lessard L, Begin LR, Karakiewicz P, Masson AM, Saad F. Nuclear factor-kappaB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer. Clin Cancer Res. 2004 Dec 15;10(24):8460-4.

26. Koumakpayi IH, Diallo JS, Le Page C, Lessard L, Filali-Mouhim A, Begin LR, et al. Low nuclear ErbB3 predicts biochemical recurrence in patients with prostate cancer. BJU Int. 2007 Jul 4;100(2):303-9.

27. Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F. Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with

clinical outcome of prostate cancer patients. Br J Cancer. 2006 Jun 19;94(12):1906-12.

28. Gannon PO, Alam Fahmy M, Begin LR, Djoukhadjian A, Filali-Mouhim A, Lapointe R, et al. Presence of prostate cancer metastasis correlates with lower lymph node reactivity. Prostate. 2006 Dec 1;66(16):1710-20.
29. Koumakpayi IH, Diallo JS, Le Page C, Lessard L, Gleave M, Begin LR, et al. Expression and nuclear localization of ErbB3 in prostate cancer. Clin Cancer Res. 2006 May 1;12(9):2730-7.
30. Lessard L, Mes-Masson AM, Lamarre L, Wall L, Lattouf JB, Saad F. NF-kappa B nuclear localization and its prognostic significance in prostate cancer. BJU Int. 2003 Mar;91(4):417-20.
31. Diallo JS, Aldejmah A, Filali Mouhim A, Alam Fahmy M, Koumakpayi IH, Sircar K, et al. NOXA and PUMA Expression Add to Clinical Markers in Predicting Biochemical Recurrence of Prostate Cancer Patients in a Survival Tree Model. Clin Cancer Res. in press.
32. Therneau T AE. An introduction to recursive partitioning using the rpart routine. Technical Report. Rocester: Section of Biostatistics, Mayo Clinic; 1997. Report No.: 61.
33. Breiman L FJ, Olshen RA, Stone CJ. Classification and regression trees. California: CRC Press; 1984.
34. Graf E, Schmoor C, Sauerbrei W, Schumacher M. Assessment and comparison of prognostic classification schemes for survival data. Stat Med. 1999 Sep 15-30;18(17-18):2529-45.
35. Team RDC. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation fro Statistical Computing; 2006.
36. Diallo JS, Peant B, Lessard L, Delvoye N, Le Page C, Mes-Masson AM, et al. An androgen-independent androgen receptor function protects from inositol hexakisphosphate toxicity in the PC3/PC3(AR) prostate cancer cell lines. Prostate. 2006 Sep 1;66(12):1245-56.
37. de Winter JA, Trapman J, Brinkmann AO, Boersma WJ, Mulder E, Schroeder FH, et al. Androgen receptor heterogeneity in human prostatic

carcinomas visualized by immunohistochemistry. J Pathol. 1990 Apr;160(4):329-32.

38. Banerjee PP, Banerjee S, Brown TR. Increased androgen receptor expression correlates with development of age-dependent, lobe-specific spontaneous hyperplasia of the brown Norway rat prostate. Endocrinology. 2001 Sep;142(9):4066-75.

39. Lei Q, Jiao J, Xin L, Chang CJ, Wang S, Gao J, et al. NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss. Cancer Cell. 2006 May;9(5):367-78.

40. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med. 2003 Jul 24;349(4):366-81.

41. Stanbrough M, Leav I, Kwan PW, Bubley GJ, Balk SP. Prostatic intraepithelial neoplasia in mice expressing an androgen receptor transgene in prostate epithelium. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10823-8.

42. Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature. 2007 Aug 2;448(7153):595-9.

43. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005 Oct 28;310(5748):644-8.

44. Ngan ES, Hashimoto Y, Ma ZQ, Tsai MJ, Tsai SY. Overexpression of Cdc25B, an androgen receptor coactivator, in prostate cancer. Oncogene. 2003 Feb 6;22(5):734-9.

45. Ceraline J, Cruchant MD, Erdmann E, Erbs P, Kurtz JE, Duclos B, et al. Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. Int J Cancer. 2004 Jan 1;108(1):152-7.

46. Coughlin SS, Hall IJ. A review of genetic polymorphisms and prostate cancer risk. Ann Epidemiol. 2002 Apr;12(3):182-96.

47. Lessard L, Karakiewicz PI, Bellon-Gagnon P, Alam-Fahmy M, Ismail HA, Mes-Masson AM, et al. Nuclear localization of nuclear factor-kappaB p65 in primary prostate tumors is highly predictive of pelvic lymph node metastases. Clin Cancer Res. 2006 Oct 1;12(19):5741-5.

48. Litvinov IV, Antony L, Dalrymple SL, Becker R, Cheng L, Isaacs JT. PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor. Prostate. 2006 Sep 1;66(12):1329-38.

49. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cell Endocrinol. 1997 Jan 3;126(1):59-73.

Table 1. Patient cohort characteristics

Overall patient cohort	
Age median (min-max)	
Cancer-free patients	35 (15-45)
PCa patients	62 (49-70)
AIPCa patients	79 (65-95)
Primary Prostate Cancer Patient Cohort	
Pathological Stage	
Stage 2	34
Stage 3	28
Invasion	
Extraprotatic extension	20
Lymph node imetastasis	9
Seminal vesicle invasion	9
Became Hormone-refractory	5
Gleason Score	
Gleason 4	8
Gleason 5	14
Gleason 6	13
Gleason 7	17
Gleason 8-9	10
Pre-operative PSA	
<10 ng	32
>10 ng	28
Not available	2
PSA relapse	
Relapse	35
No relapse	27
Surgical margins	
Negative	31
Positive	31
Deaths within follow-up period	9

Table 2. AR expression in correlation with clinico-pathological features

	Cytoplasmic AR	Cytoplasmic AR categorized *	Nuclear AR	Nuclear AR categorized **	Nuclear / Cytoplasmic AR ratio	Nuclear / Cytoplasmic AR ratio categorized ***
age	0.002 p=0.989	-0.103 p=0.424	-0.009 p=0.943	-0.002 p=0.998	-0.007 p=0.959	0.117 p=0.367
pre-operative PSA	0.210 p=0.108	0.07 p=0.597	-0.030 p=0.819	-0.100 p=0.448	-0.223 p=0.086	-0.106 p=0.421
Margin	**-0.431** **p<0.0001**	**-0.590** **p<0.0001**	**-0.297** **p=0.019**	**-0.315** **p=0.013**	**0.356** **p=0.005**	**0.49** **p<0.0001**
Stage	-2.11 p=0.100	**-0.354** **p=0.005**	-0.100 p=0.440	-0.170 p=0.186	0.172 p=0.181	**0.294** **p=0.02**
Gleason	0.058 p=0.656	-0.001 p=0.994	0.011 p=0.935	-0.112 p=0.386	-0.085 p=0.509	-0.110 p=0.396
Lymph node invasion	-0.217 p=0.091	**-0.280** **p=0.027**	-0.114 p=0.377	-0.123 p=0.340	0.219 p=0.088	**0.262** **p=0.04**
Extra-capsular invasion	-0.174 p=0.176	**-0.303** **p=0.017**	-0.044 p=0.732	-0.140 p=0.278	0.187 p=0.145	**0.361** **p=0.004**
Seminal vesicle invasion	-0.07 p=0.588	-0.103 p=0.426	-0.086 p=0.505	-0.057 p=0.659	-0.005 p=0.967	-0.067 p=0.606

Table 2. Correlation between cytoplasmic, nuclear, nuclear/cytoplasmic AR expression, and clinico-pathological parameters. Non-parametric Spearman rank tests were used to assess correlation coefficients (top numbers). Cytoplasmic, nuclear and nuclear/cytoplasmic AR expression were used either as continuous variables or as binary variables (*low=cytoplasmic AR <2.1 , **low=nuclear AR < 7.3, ***low=nuclear/cytoplasmic AR <3.5, see materials and methods). A negative number indicates that increasing AR expression was associated with decreasing status (e.g. low age, low PSA, negative margins, low stage, low Gleason, no lymph node, extracapsular, or seminal vesicle invasion). Significant correlations are highlighted in bold ($p<0.05$). All cutoffs were obtained using RPART.

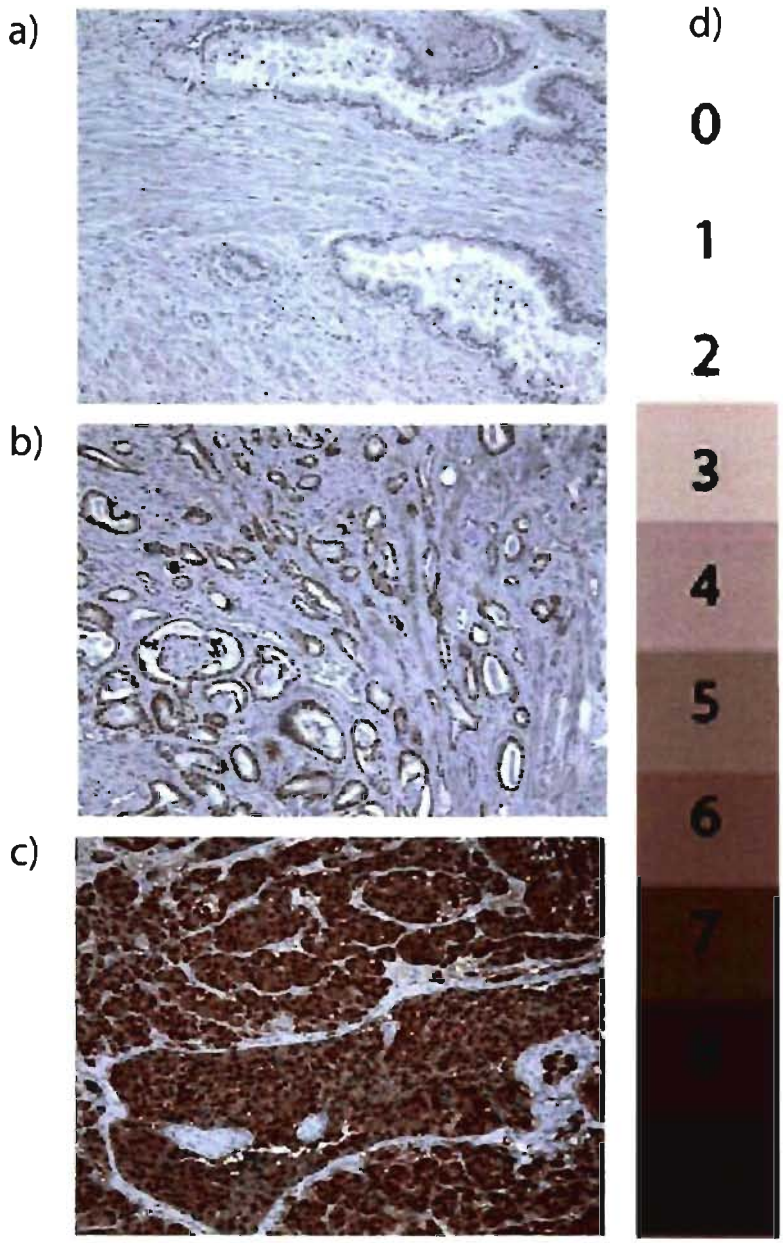
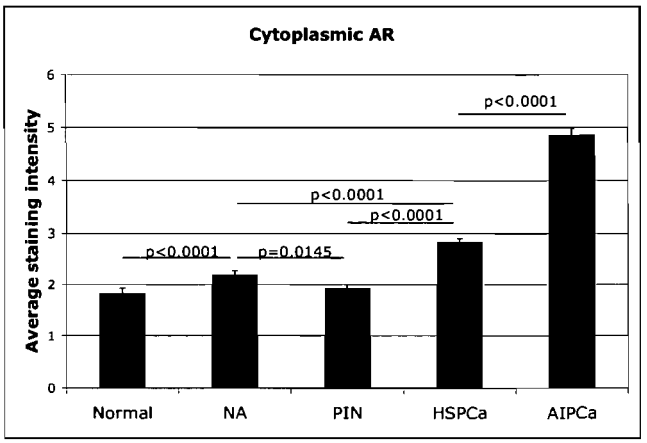


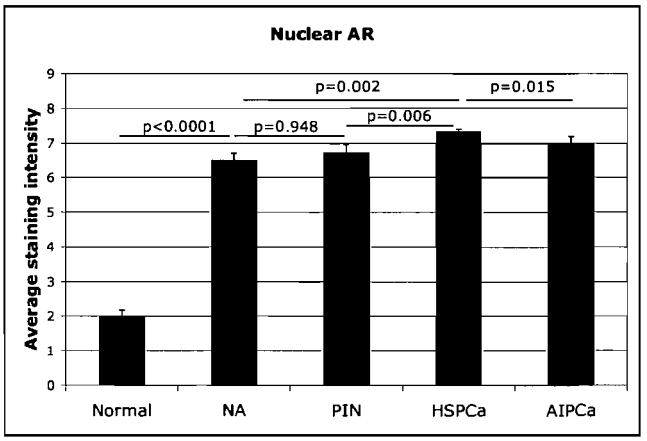
Figure 1. AR expression and sub-cellular localization in prostate tissues

Figure 1. Immunohistochemical staining of paraffin-embedded prostate tissues using AR-specific antibody. An antibody recognizing the AR was used to stain A) Normal prostate tissue from cancer-free patients. B) Hormone-sensitive prostate carcinomas. C) Hormone-refractory TURP specimens. Note the increased intensity of cytoplasmic staining in hormone-refractory samples. D) Color scale standard used for the evaluation of images obtained from tissue microarrays. Scale was constructed from several digital pictures evaluated in the study as described in materials and methods. Numeric values correspond to the associated intensity score.

a)



b)



c)

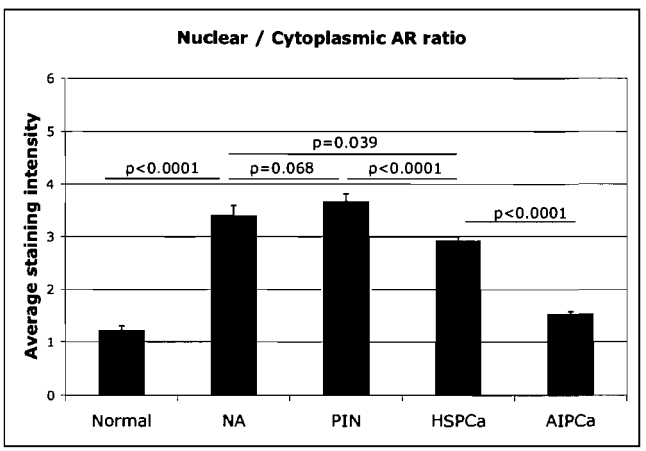


Figure 2. Average cytoplasmic, nuclear and nuclear / cytoplasmic AR staining intensity ratio in prostate specimen subtypes

Figure 2. Average cytoplasmic, nuclear and nuclear / cytoplasmic AR staining intensity ratio in prostate specimen subtypes. A) Cytoplasmic AR expression. B) Nuclear AR expression. C) Nuclear / cytoplasmic AR ratio. Normal = normal prostate tissues obtained from cancer-free patients (n=96 cores). NA=non-neoplasic tissue found adjacent to HSPCa (n=92 cores). PIN = Prostatic intraepithelial neoplasia (n=44 cores), HSPCa= hormone-sensitive prostate cancer (n=227 cores), AIPCa= androgen-independent prostate cancer specimens obtained by TURP (n=157). Kruskal-Wallis non-parametric test was used to assess significance of observed differences.

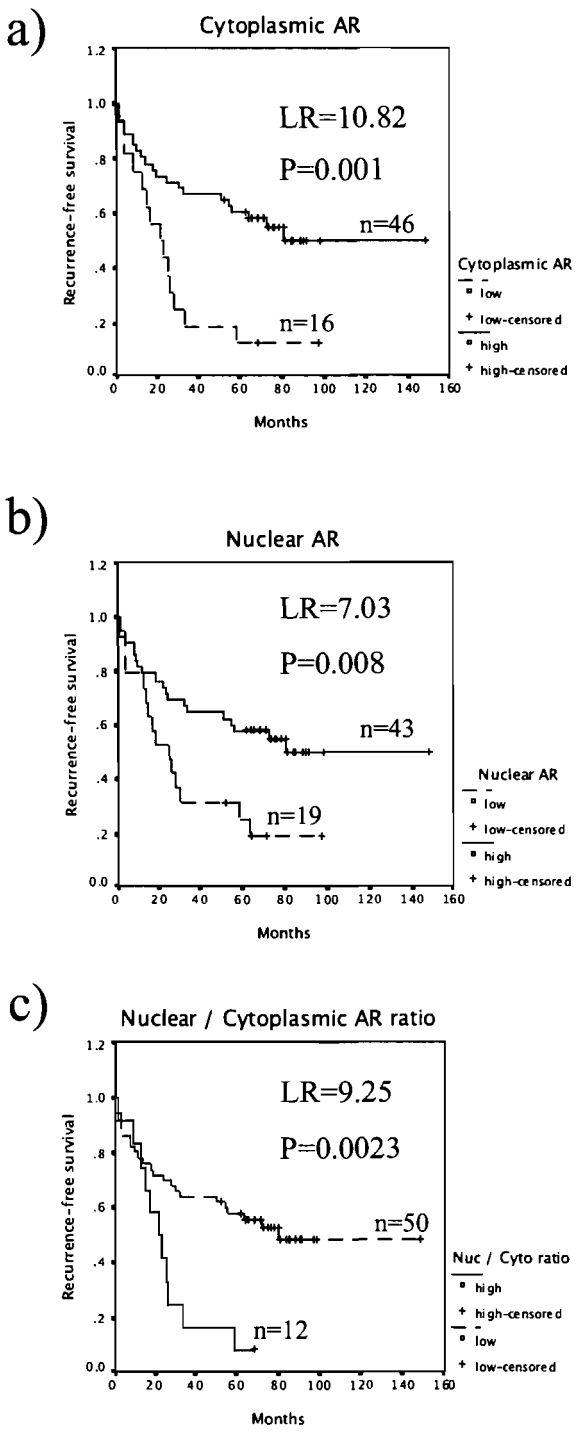


Figure 3. AR expression and sub-cellular localization in relation to biochemical recurrence following radical prostatectomy.

Figure 3. Prediction of biochemical recurrence using cytoplasmic, nuclear and nuclear/cytoplasmic AR staining intensity ratio. A) Low cytoplasmic AR is associated to an early onset of BCR. Low (dashed) indicates cytoplasmic AR expression below 2.1 and high (full) indicates cytoplasmic AR expression over or equal to 2.1. B) Low nuclear AR is associated to an early onset of BCR. Low (dashed) indicates nuclear AR expression below 7.3 and high (full) indicates nuclear AR expression over or equal to 7.3. B) High nuclear / cytoplasmic AR ratio is associated to an early onset of BCR. Low (dashed) indicates nuclear / cytoplasmic AR expression ratio below 3.5 and high (full) indicates nuclear AR expression over or equal to 3.5.

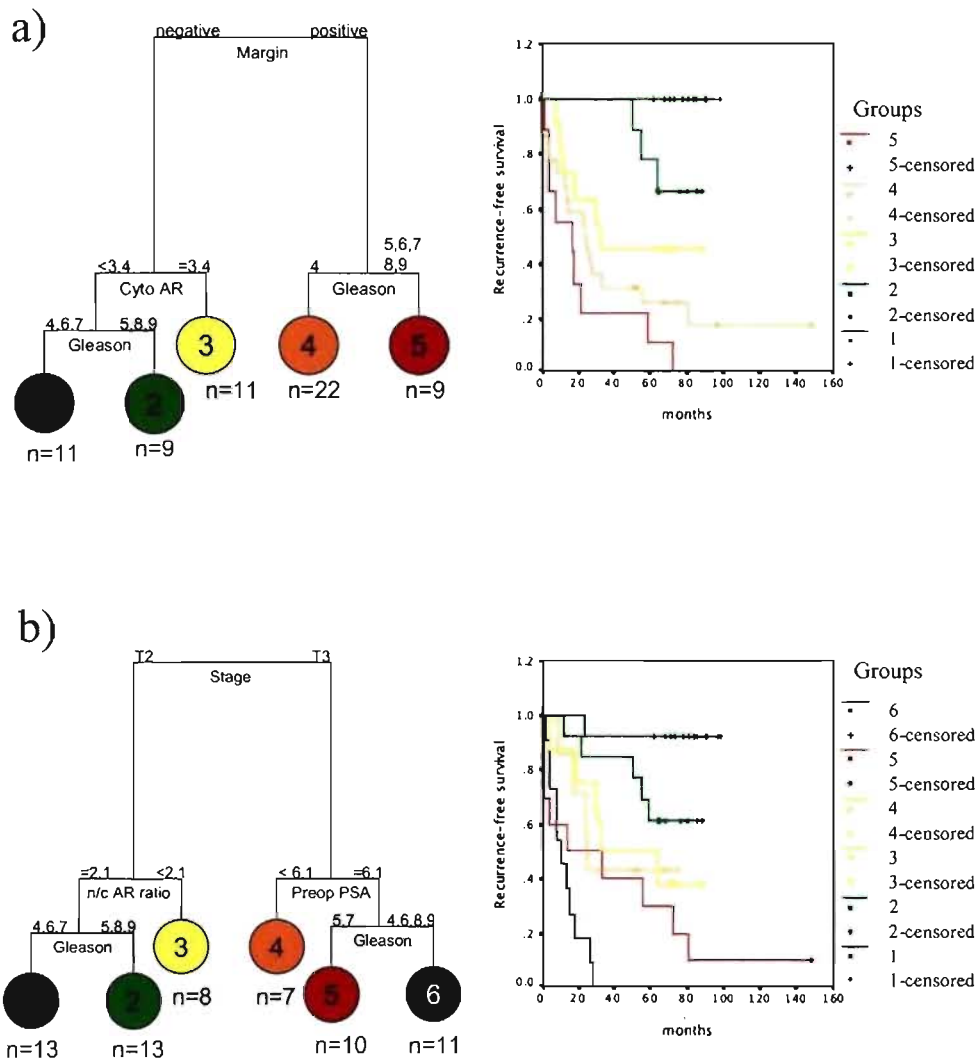


Figure 4. Two highest ranking survival tree models

Figure 4. Top–ranking RPART survival tree models for prediction of BCR and associated Kaplan-Meir plots. A) Rank #1. IBS=0.118 (95% CI=0.081-0.144). Cyto AR=cytoplasmic AR expression B) Rank #2. IBS=0.122 (95%CI=0.08-0.14). n/c AR ratio= nuclear / cytoplasmic AR staining intensity ratio. Colored circles within the survival trees represent the different prognostic groups and are depicted in the associated Kaplan-Meier plots represented by curves of the same color (and number). Below each colored circle, n indicates the number of patients in each group.

CHAPTER V

Inositol hexakisphosphate and Proteasome Inhibitors Elicit Enhanced Mitochondrial Depolarization in Androgen-Independent Prostate Cancer Cells: Implication of BCL-2 family proteins

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Author contributions

I wrote this manuscript and performed most of the experiments presented. However, all co-authors were involved in the editing process. Blandine Betton played a major role in producing Figure 6. The mitochondrial depolarization assays were done with the help of Nicolas Parent. Benjamin Péant helped on several fronts, notably with the optimization of western blotting conditions. Laurent Lessard's help and expertise was pivotal in the production of Fig 1a.

Keywords: Inositol Hexakisphosphate, Proteasome Inhibitors, Prostate Cancer, BCL-2 Family Proteins.

Abstract

Effective treatments for androgen-independent prostate cancer (AIPCa) are lacking. To address this, emerging therapeutics such as proteasome inhibitors are currently undergoing clinical trials. Inositol hexakisphosphate (IP6) is an orally non-toxic phytochemical that exhibits anti-tumor activity against several types of cancer including prostate cancer (PCa). We have previously shown that treatment of PC3 cells with IP6 induces the transcription of a subset of NF-κB-responsive and pro-apoptotic BCL-2 family genes. In this study we report that although NF-κB subunits p50/p65 translocate to the nucleus of PC3 cells in response to IP6, inhibition of NF-κB-mediated transcription using non-degradable IκB-α does not modulate IP6 sensitivity. Treatment with IP6 also leads to increased protein levels of PUMA, BIK/NBK and NOXA between 4 and 8 hours of treatment and decreased levels of MCL-1 and BCL-2 after 24-hours. Although blocking transcription using actinomycin D does not modulate PC3 cell sensitivity to IP6, inhibition of protein translation using cycloheximide has a significant protective effect. In contrast, blocking proteasome-mediated protein degradation using MG-132 significantly enhances the ability of IP6 to reduce cellular metabolic activity in both PC3 and DU145 AIPCa cell lines. This effect of combined treatment on mitochondrial depolarization is particularly striking and is also reproduced by another proteasome inhibitor (ALLN). The enhanced effect of combined MG132/IP6 treatment is almost completely inhibited by cycloheximide, and correlates with changes in BCL2-family protein levels. Altogether these results suggest a role for BCL-2 family proteins in mediating the combined effect of IP6 and proteasome inhibitors and warrant further pre-clinical studies for treatment of AIPCa.

Introduction

Prostate cancer (PCa) remains a leading cause of cancer-related death in North-American men (1). Though localized forms of the disease can often be successfully treated by surgery or radiotherapy, a significant proportion of patients having undergone such interventions are at risk of disease recurrence. Androgen deprivation therapy can prolong the life expectancy of these patients; however, androgen-independent (AI) PCa eventually arises. As currently available treatment options for AIPCa are lacking, there is a growing need for novel therapeutics that can be effective against this advanced stage of the disease.

Inhibitors of the proteasome are showing promise as anti-cancer agents against several cancers. Although the proteasome harbors active sites for several types of proteolytic activity, these inhibitors generally consist of small synthetic peptides targeting the chymotryptic activity of the proteasome, which is thought to be a rate-limiting catalytic step in proteasome-mediated protein degradation (2). Notably, one proteasome inhibitor (bortezomib) has been recently approved by both the Food and Drug Administration (FDA, USA) and the European Agency for Evaluation of Medicinal products (EMEA) for treatment of recurrent multiple myeloma and is currently undergoing clinical trials for AIPCa (3-6).

Some studies have suggested that a major downstream target of proteasome inhibition is nuclear factor κB (NF-κB), a transcription factor involved in the transcriptional regulation of hundreds of genes implicated in cell proliferation, differentiation and cell survival (7). NF-κB is a dimeric protein composed of hom or hetero-dimers of p50, p65 (RelA), p52, RelB and c-Rel subunits. NF-κB activity is directly regulated by the action of inhibitor of κB (IκB) proteins (e.g. IκB-α, β, ε, p100) and IκB kinases (8-10). IκBs retain NF-κB in the cell cytoplasm and diminish its transactivation potential whereas IκB kinases phosphorylate both IκBs as well as NF-κB itself. Importantly, phosphorylation of IκBs by IKKs leads to their proteasome-mediated degradation and to the release and nuclear translocation of NF-κB subunits (classically p50/p65). Alternately, in what is referred to as the non-canonical pathway, the

proteasome catalyses the processing of p100 into the NF-κB p52 subunit, which may also translocate to the nuclear compartment (7, 10, 11).

More recently, proteasome inhibitors have also been found to up-regulate the expression of BCL-2 family proteins. Notably, pro-apoptotic BH3-only proteins such as NOXA, BIK/NBK, BIM, and PUMA see their levels increased following treatment with proteasome inhibitors such as MG-132, ALLN, Lactacystin, and Bortezomib (12-16). In parallel, anti-apoptotic proteins such as MCL-1 can also be up regulated following proteasome inhibition, leading investigators to combine proteasome inhibitors with strategies aimed at thwarting the anti-apoptotic response with some success (13).

BCL-2 family proteins play a major role in the control of mitochondrial permeability. Importantly, mitochondrial outer-membrane permeation (MOMP) is a key event in cell death whether by means of apoptosis or necrosis (17). Although the role of pro-apoptotic proteins BAX and BAK in this process is clear, the nature of how these are activated to initiate MOMP is currently under debate. In particular, how BH3-only BCL-2 family proteins initiate BAX/BAK-assisted mitochondrial permeability pore formation remains unclear. It has been suggested that a subset of BH3-only proteins act as sensitizers to the action of other BH3-only proteins thought to play the role of BAX/BAK activators (18). Other recent evidence suggests that BH3-only proteins such as NOXA, BAD, and BIK/NBK, may act as inactivators of anti-apoptotic BCL-2/BCL-XL and MCL-1, preventing their inhibitory interaction with activator BH3-only proteins such as PUMA, BID and BIM (19). In either event, it is clear that the increased activity of pro-apoptotic BH3-only proteins is important for the initiation of cell death by various stimuli.

Inositol hexakisphosphate (IP6) is a naturally occurring phytochemical abundant in soy and legumes that exhibits anti-cancer activity in a wide range of cancers (20). Although definite mechanisms have yet to be established, IP6 activity has been reported to involve several processes (21). In DU145 AIPCa cells, IP6 has been suggested to inhibit phosphatidyl inositol-3-kinase (PI3K), prevent epidermal growth factor receptor (EGFR) signaling through the mitogen

activated protein kinase (MAPK) signaling cascade (22), and diminish constitutive NF-κB activity (23). Still in the context of DU145 cells, IP6 was found to modulate cdk-cyclin and pRb/E2F complexes leading to p21 and p27 up-regulation and cell cycle arrest in G1 (24).

Treatment of AIPCa cells with IP6 can also induce classic hallmarks of apoptotic death such as caspase-3 activation, cleavage of poly ADP-ribose polymerase (PARP), increased cell-surface phosphatidyl-serine and DNA fragmentation (24, 25). In LNCaP cells, treatment with IP6 was found to increase the expression of pro-apoptotic BAX while decreasing levels of anti-apoptotic BCL-2(26). In addition, we have recently shown that in PC3 cells, IP6 induces the mRNA expression of *PUMA*, *NOXA* and *BAX* as well as a subset of NF-κB-responsive genes including *IκB-α* and *IRF-2* (25). As such, we hypothesized that the up-regulation of NF-κB-responsive genes as well as of genes coding for pro-apoptotic proteins could play a role in mediating the pro-apoptotic effects of IP6 in PC3 cells. In this study, we investigated the role of NF-κB and BCL-2 family members in IP6-induced cell death. We also evaluated whether IP6 could be useful in combination with proteasome inhibitors.

Materials and methods

Cell culture

22Rv1, PC3, LNCaP and DU145 were obtained from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 complemented with 10% fetal calf serum, gentamicin (50 μg/ml) and amphotericin B (250 ng/ml) (Gibco-BRL, MD, USA). Cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere and sub-cultured at 1:3 (LNCaP), or 1:5 (PC3, 22Rv1, DU145) by trypsinization with 0.25% trypsin for 5-10 minutes at 37°C (Gibco-BRL, MD, USA).

Drugs and inhibitors

IP6 *(Myo*-Inositol hexakisphosphate dodecasodium salt, Sigma-Aldrich, USA) was kept as a 100 mM stock solution diluted in water. Actinomycin D (Sigma-Aldrich, USA) was dissolved in DMSO and kept as a 1 mg/ml stock solution.

Cycloheximide (Supelco, PA, USA) stock solution (50 mg/ml) was dissolved in ethanol. MG-132 (Calbiochem, USA) was also diluted in ethanol as a 20 mM stock solution. ALLN (Calbiochem, USA) was reconstituted in DMSO and kept at a stock concentration of 10 mM.

Cell seeding and treatments

For WST-1 assays, whole cell extracts, nuclear/cytoplasmic extracts, RNA extractions and JC-1 assays, a similar cell seeding procedure was used. Briefly, cells were trypsinized and counted on a hemocytometer, then diluted in the appropriate media at 200 000 cells/ml and distributed in 96-well plates (100 µl/well for WST-1 and Luciferase reporter assays) or 6-well plates (2 ml/well for JC-1 assay and RNA extractions). For whole cell and nuclear/cytoplasmic extracts, cells (at a density of 200 000 cells/ml) were seeded in 60 mm petris (3 ml/Petri), 100 mm petris (5ml/petri), or 150 mm petris (10 ml/petri) petris. Cells were allowed to adhere overnight prior to treatment. For the experiments assessing the effects of dominant-negative (DN) IκB-α on IP6 efficacy, cells were transfected with DN-IκB-α (pCMV-IκB-αM, Clontech, CA, USA) or control plasmid pCMV-Neo (Clontech, CA, USA) 24-hours prior to seeding. Efficacy of DN-IκB-α transfection was verified in parallel by luciferase assay (see below). Cells were then treated with the indicated concentrations of IP6. In the experiments where the effect of actinomycin D, cycloheximide, MG-132, cycloheximide + MG-132, and ALLN on the activity of IP6 was assessed, cells were pre-treated 4 hours prior to addition of IP6.

WST-1 metabolic assay

After a 24-hour treatment with IP6 (in addition to treatment with the appropriate inhibitors where indicated), 10 µl of WST-1 reagent (Roche, USA) was added to wells and plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. Incubation times were optimized for each cell line as recommended by manufacturer. WST-1 signal was measured on a Bio-Rad Model 3550 microplate reader at 450 nm with reference at a wavelength of 655 nm. Following data

acquisition, cell metabolic activity was calculated by first subtracting the readout of the WST-1 background (media + WST-1) from all values. For each independent experiment, the median of the replicates was calculated for each treatment. Subsequently, relative metabolic activity was calculated as being the median WST-1 readout of the drug treated well / median WST-1 readout of vehicle-treated well. In the cases where cells were pre-treated with an inhibitor (actinomycin D, cycloheximide, or MG-132), values were normalized relative to the inhibitor-treated well that was not challenged with IP6. Cellular metabolic activity was plotted as a function of IP6 concentration.

Protein extracts

Cells were scraped in their media, collected by centrifugation and washed twice with cold PBS. Pellets were frozen at -80°C. Subsequently, whole cell extractions were performed by applying cold lysis buffer (10 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 0.5% NP-40, 10 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 10 μmol/L phenylmethylsulfonyl fluoride, 200 μmol/L orthovanadate) on ice for 30 min. Whole cell extracts were collected after centrifugation in a Heraeus Biofuge (13,000 rpm for 10 min at 4°C) and were immediately stored at -80°C. Protein concentration was measured by Bradford assays (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions.

Nuclear and cytoplasmic protein extracts

After cell treatments, media was aspirated, cells were scraped and washed twice with cold PBS, and pellets were frozen at -80°C. Ice-cold buffer I (10 mmol/L HEPES, 50 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L MgCl2) with freshly added protease and phosphatase inhibitors (10 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 10 μmol/L phenylmethylsulfonyl fluoride, 200 μmol/L orthovanadate) was added and cells were incubated on ice for 30 min. Cell membranes were lysed by incubating with 1% NP40 for 10 min. Cytosolic fractions were collected after centrifugation (3,000 x g for 5 min at 4°C). Ice-cold

buffer II (10 mmol/L HEPES, 400 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5 mmol/L DTT) with freshly added protease and phosphatase inhibitors was then added to the nuclear aggregates and incubated on ice for 1 h. Nuclear protein fractions were collected after centrifugation (14,000 x g for 15 min at 4°C). Each fraction was immediately stored at -80°C.

Antibodies

Antibodies recognizing NOXA (OP180) and PUMA (PC686) were obtained from Calbiochem (USA). Antibodies detecting BAX (N-20, sc-493), BCL-2 (C-2, sc-7382), BIK/NBK (N-19, sc-1710), MCL-1 (H-260, sc-20679), RAN (C-20, sc-1156), IκB-α (C-21, sc-371), NF-κB p65 (F-6, sc-8008), α-TUBULIN (TU-02, sc-8035), as well as horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (CA, USA). The anti-NF-κB p50 (06-886) antibody was purchased from Upstate (NY, USA). Antibodies recognizing β-ACTIN (AC-15, ab6276) and GAPDH (ab-9485) were obtained from Abcam (MA, USA).

Western blot analysis

For Western blot analyses, 20-75 μg of whole cell protein or nuclear/cytoplasmic protein extracts were resolved on 10-17% polyacrylamide gels and then transferred onto nitrocellulose or PVDF membranes at 60V for 1 to 2 hours. Blots were blocked using 5% non-fat dry milk in PBS-Tween 0.1% buffer for 2 hours at room temperature. Membranes were then probed using antibodies recognizing PUMA (1:1000), NOXA (1:200), BAX (1:1000), MCL-1 (1:100), BIK/NBK (1:200), BCL-2 (1:200), RAN (1:3000), IκB-α (1:1000), NF-κB p65 (1:750), NF-κB p50 (1:750), α-TUBULIN (1:1000), β-ACTIN (1:10000) and GAPDH (1:5000). Membranes were then incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Life Sciences Inc., Arlington Heights, IL) in blocking buffer for 45 minutes at room temperature and developed with enhanced chemiluminescence (ECL) substrate (Amersham Life Sciences Inc., Arlington Heights, IL). When necessary, membranes were stripped using the

protocol described in the ECL kit (Amersham Life Sciences Inc., Arlington Heights, IL) and re-probed. Densometric analysis was done using the BioRad Quantity One® software (CA, USA).

Quantitative Real time PCR

After the indicated times of treatment with IP6 and/or actinomycin D, media was removed and RNA was extracted with Trizol reagent according to manufacturers' instructions (Invitrogen, ON, Canada). Concentration of RNA samples was determined using a Beckman DU-600 spectrophotometer. 2 μg of RNA was used to synthesize a cDNA using the SuperScript first-strand synthesis system (random hexamer method) according to manufacturers' instructions (Invitrogen, ON, Canada). The QuantiTect SYBR Green PCR kit was used as recommended (Qiagen, ON, Canada). Real time PCR reactions were performed on a Rotor-gene RG-300 (Corbett Research, Australia). Optimal threshold and reaction efficiency were determined using the Rotor-gene software. Melt curves for each primer exhibited a single peak, indicating specific amplification, which was also confirmed by agarose gel. Ct values were determined using the Rotor-gene software at the optimal threshold previously determined for each primer. Relative IκB-α/actinB ratios were calculated using the Pfaffl method (27). Fold induction was calculated relative to the vehicle treated control. Experiments were done twice and real time measurements were done in duplicate. Primer sequences used were described previously (25).

JC-1 mitochondrial depolarization assay

PC3 cells were incubated with 10 μg/ml of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanide iodide, Molecular Probes, OR, USA) at 37°C in a humidified 5% CO_2 atmosphere for 15 minutes. Cells were washed twice with PBS and trypsinized with 500 μl 0.25% trypsin for 10 minutes. Cells were then collected and put in fluorescence activated cell sorting (FACS) counting tubes containing 500 μl RPMI 1640 complemented with 10% FCS. JC-1 emission was measured in individual cells using a Coulter Epics® XL-MCL. The

fold change in cells presenting de-polarized mitochondria (green-shift in JC-1 fluorescence) was calculated relative to vehicle-treated control for each time point.

Luciferase Reporter Assays.

PC3 cells were seeded at 400 000 cells/ml in 60 mm petris (3 ml). The following morning, lipofectamine PLUS reagent (Invitrogen, ON, Canada) was used to transfect 1 μg pCMV-IκB-αM (or pCMV-Neo control), 0.8 μg of a 3enh-κB-ConA-luc green luciferase reporter (or control ptkGL3 luciferase gene driven by a minimal thymidine kinase promoter), and 0.2 μg of a pCMV-RL Renilla luciferase reporter (Promega, WI, USA). The following day, transfected cells were re-seeded at a density of 20 000 cells per well in 96-well plates. To assess the effects MG-132 on κB-luc activity, transfected cells were then treated with MG-132 for 24 hours after which time green and renilla luciferase activity was assayed using the dual luciferase reporter assay system (Promega, WI, USA) on a Victor^3 luminometer/fluorimeter (Perkin Elmer, ON, Canada). To calculate the relative κB reporter activity, green luciferase activity was first normalized according to renilla luciferase output (transfection efficacy control) then corrected for background effects by subtracting the basal activity detected from the ptkGL3 plasmid. The 3enh-κB-ConA-luc (hereby refered to as κB-luc), carrying a firefly luciferase gene under the control of a trimer of κB consensus was a kind gift from Dr. Juana Wietzerbin (Institut Curie, Paris, France). To construct the ptkGL3 control plasmid, the SV40 promoter (BglII/HindII fragment) was removed from the pGL3 basic plasmid (Promega, WI, USA) and a minimal thymidine kinase promoter (BglII/HindIII fragment) from the pNF-κB-d2EGFP plasmid (Clontech, USA) was inserted in its place.

Statistics

Averages, standard errors of the mean, and p-values (calculated using T-test) were calculated from individual experiments. The numbers of replicates as well as the number of independent experiments are indicated in the figure legends. All

statistical tests were performed in Excel (Microsoft®, WA, USA). For WST-1 assays, an effect was deemed significant when a p-value <0.05 was reached for at least one concentration of IP6.

Results

IP6 induces NF-κB nuclear translocation in PC3 cells

We have previously observed that a subset of NF-κB responsive genes is up regulated in response to IP6 in PC3 cells. Notably, we have found that *IκB-α* mRNA levels can increase up to 20 fold following a 24-hour treatment with 2 mM IP6 (25), beginning as early as 4 hours post-treatment (data not shown). As *IκB-α* is thought to be regulated by p50/p65 NF-κB subunits (28), we set out to determine the effects of IP6 of NF-κB subunit nuclear translocation. Fig. 1a shows that although only minimal changes in the status of p52 and its precursor p100 could be observed, nuclear p50 and p65 increased substantially following a 24 hour treatment with IP6 (Fig. 1a), an event that was visible as early as 4 hours following challenge with IP6 (data not shown). Notably, the increase in nuclear p50/p65 was not due to contamination from cytoplasmic proteins as evidenced by the absence of α-TUBULIN in the nuclear extracts (Fig.. 1a). In contrast to observations at the mRNA level (25), a slight decrease in IκB-α protein (which was primarily located in the cytoplasm), was detected in parallel (Fig.. 1a and 3). Overall, these data indicated that canonical NF-κB subunits translocate to the nucleus in response to treatment with IP6.

Reduced NF-κB transcriptional activity by expression of a non-degradable IκB-α does not modulate the response to IP6.

Others have reported that in DU145 cells, NF-κB nuclear translocation decreases in response to IP6, resulting in reduced NF-κB DNA-binding activity (23). Consequently, the observation that NF-κB p50 and p65 translocate to the nucleus in response to IP6 in PC3 cells lead us to ask whether the modulation of NF-κB-mediated transcription was implicated in the activity of IP6 in PC3 cells. To address this, we transfected PC3 cells with a non-degradable dominant-

negative mutant of IκB-α (29) (DN-IκB-α) or a control vector (pCMVNeo) and determined whether this could modulate the effects of IP6. As shown in Fig. 1b, transient transfection of DN-IκB-α effectively reduced NF-κB transcriptional activity relative to the pCMVNeo control as assessed using the κB-luc reporter. However, WST-1 assays indicate that DN-IκB-α and pCMVNeo-transfected PC3 cells respond similarly to a challenge with IP6 (Fig.. 1c), suggesting that NF-κB transcriptional activity does not correlate with IP6 cytotoxicity.

IP6 modulates BCL-2 family protein expression

In parallel with NF-κB-responsive genes, we have previously reported that the mRNA levels of BCL-2 family pro-apoptotic genes such as NOXA, PUMA and BAX increase in response to IP6 [25].Hence, we next set out to determine how the protein levels of these pro-apoptotic BCL-2 family proteins changed in time following treatment with IP6. To address this, PC3 cells were challenged with 2 mM IP6 and whole cell extracts were prepared after 4, 8 and 24 hours of continuous treatment. The extracts were then probed by western blot. As shown in Fig. 2, the expression of PUMA increased as early as 4 hours following treatment with IP6 until at least 8 hours post-treatment. Similarly, increased NOXA expression was also observed although later (8 hours) and to a lesser extent. Both PUMA and NOXA expression subsequently returned to normal after 24 hours of treatment with IP6. In contrast, no clear changes in BAX protein expression were observed. We also looked at whether protein expression of other pro and anti-apoptotic BCL-2 family members could be modulated in response to IP6. As can be seen in Fig. 2, BIK/NBK expression is also up regulated by IP6 similarly to PUMA though to a lesser extent, particularly at 4 hours. In contrast, we could observe decreased expression of anti-apoptotic proteins MCL-1 and BCL-2, though only after 24 hours of treatment with IP6. These data suggested that IP6 induces temporal changes in the ratio of pro and anti-apoptotic BCL-2 family proteins.

Protein synthesis is important for IP6-induced cytotoxicity.

In light of the results presented in Fig. 2 in addition to our observation of increased mRNA expression of pro-apoptotic BCL-2 family members in previous study (25), we next set out to determine whether transcriptional up regulation of pro-apoptotic genes such as *PUMA*, *NOXA* and potentially *BIK/NBK* could be important for mediating the effects of IP6. To gain an overall appreciation of this, we compared the efficacy of IP6 in PC3 cells pre-treated (4 hours) with an inhibitor of mRNA transcription (actinomycin D(30)) or vehicle (DMSO). Fig. 3a shows that continuous treatment with actinomycin D starting 4 hours prior to addition of IP6 (for 24 hours) did not modulate PC3 cell sensitivity to IP6. To verify that actinomycin D effectively blocked mRNA synthesis in our conditions, we looked at the mRNA levels of *IκB-α* in response to IP6. Fig. 3b shows that IP6-induced IκB-α up-regulation after 24 hours of treatment was effectively blocked by actinomycin D but not by DMSO. Because IP6 treatment could lead to protein up-regulation independently of transcription, we next assessed the efficacy of IP6 in PC3 cells where protein production was blocked using cycloheximide (50 μg/ml, 4h pre-treatment). Fig. 3c shows that a continuous treatment with the translation inhibitor cycloheximide (30) starting 4 hours prior to treatment with IP6 significantly protected from IP6-induced toxicity ($p<0.05$ at 1.5mM IP6). These data suggested a role for protein synthesis in mediating the effects of IP6 and that one possible effect of blocking protein translation would be to hinder on the temporal shift of the ratio between pro-apoptotic to anti-apoptotic protein induced by IP6.

A proteasome inhibitor sensitizes androgen-independent prostate cancer cells to the effects of IP6

Because blocking protein translation reduced the efficacy of IP6, potentially by preventing increases in pro-apoptotic protein levels, we rationalized that blocking proteasome-mediated protein degradation could have the opposite effect and instead enhance the effect of IP6. To test this hypothesis, we treated

PC3 AIPCa cells with the commonly used proteasome inhibitor MG-132 (20 μM) (31), beginning 4 hours prior to the addition of IP6, and assessed the efficacy of IP6 using WST-1 assays. Fig. 4a shows that over 24 hours, cells co-treated with MG-132 were significantly more sensitive to IP6 treatment than control cells ($p<0.005$, IP6 1mM). As inhibitors of the proteasome have been reported to inhibit NF-κB transcriptional activity, we also assessed the effect of MG-132 on κB-luc reporter activity in PC3 cells. We found that, at least in the PC3 cell line, MG-132 did not inhibit NF-κB-mediated-transcription of the κB-luc reporter. In fact, a slight increase in κB-luc reporter activity was detected with increasing doses of MG-132 (Fig. 4b). We then looked at whether MG-132 could increase the sensitivity of IP6 in other PCa cell lines. Fig. 4c shows that MG-132 co-treatment also significantly sensitized DU145 AIPCa cells to the effects of IP6 ($p<0.01$, IP6 1mM). In contrast, MG-132 did not sensitize androgen-responsive LNCaP or 22Rv1 cells to the effects of IP6 (Fig. 4e).

IP6 and proteasome inhibitors elicit enhanced mitochondrial depolarization in a protein translation-dependent fashion.

The potential involvement of one or more members of the BCL-2 family proteins in the observed effect of IP6 next prompted us to look at whether a treatment with IP6 and/or proteasome inhibitors initiated mitochondrial outer membrane permeation (MOMP). To address this, we stained both treated and control PC3 cells with JC-1 dye, and used FACS to measure the percentage of cells exhibiting a green shift in JC-1 fluorescence in treated cells relative to the control at various times following treatment. Notably, a JC-1 fluorescence shift from red to green is indicative of cells having undergone MOMP (32). We found that IP6 (2 mM) used alone caused a modest but time dependent increase in MOMP (Fig. 5a-c). As we had observed that cycloheximide protected from the effects of IP6 using WST-1 assays, we looked at whether cycloheximide (50 μg/ml) could protect from IP6-induced MOMP. We found that cycloheximide on its own could induce MOMP to an extent similar to what was observed for IP6

(Fig. 5a, c). However, co-treatment with IP6 and cycloheximide did not further increase the amount of cells exhibiting a green shift in JC-1 fluorescence (Fig. 5a, c). Similarly, we found that MG-132 used alone also caused a time-dependent increase in green-shifted JC-1 stained cells. Although likely due to the extra 4 hours of pre-treatment with MG-132, at 8 hours (plus 4 hours "pre-treatment"), MG-132 appeared to induce roughly twice the relative amount of MOMP as compared to IP6 (Fig., 5a-c). Importantly, co-treatment with MG-132 and IP6 caused a drastic increase in cells presenting MOMP. After 8 hours, cells co-treated with IP6 and MG-132 had nearly 3-fold the quantity of cells exhibiting MOMP as compared to MG-132 alone and 6-fold the quantity of cells exhibiting MOMP as compared to IP6-treated cells (Fig. 5a-c). We next questioned whether the observed enhanced effect of combined IP6 and MG-132 at the level of MOMP was specific to MG-132 or whether it could also be observed with other proteasome inhibitors. Fig. 5b shows that ALLN (10 μM) (33) also enhances the induction of MOMP when combined with IP6 in PC3 cells. In order to assess whether the enhancement of MOMP elicited by combined treatment with IP6 and proteasome inhibitors required de novo protein synthesis, we co-treated cells with cycloheximide (50 μg/ml), MG-132 (20 μM) and IP6 (2mM). We found that although addition of cycloheximide did not reduce MG-132 induced MOMP (Fig. 5a, c), addition of cycloheximide almost completely blocked the enhanced MOMP observed in response to combined treatment with MG-132 and IP6 (Fig. 5c).

MG-132 and Cycloheximide modulate the expression of BCL-2 family proteins in PC3 cells

As blocking protein translation prevented MOMP in response to combined IP6 and MG-132 treatment, we deemed it relevant to look at how BCL-2 family member expression was modulated in response to MG-132 and cycloheximide with and without IP6 (added 4 hours later). Western blotting of whole cell extracts was used to detect the expression of BCL-2 family members as well as IκB-α.

The results presented in Fig. 6 suggest that treatment of PC3 cells with MG-132 substantially increased the levels of NOXA and to a lesser extent of BIK/NBK, PUMA and MCL-1. The addition of IP6 led to a slight further increase in the levels of NOXA, BIK/NBK and MCL-1. In contrast, the treatment of PC3 cells with cycloheximide on its own caused almost complete loss of NOXA expression and substantially decreased BIK/NBK and MCL-1 protein levels. Interestingly, subsequent addition of IP6 curtailed the decrease in the levels of MCL-1 but not that of NOXA or BIK/NBK. Cycloheximide did not modulate the expression of PUMA or BAX when used alone although notably, it did not completely block IP6-induced up-regulation of PUMA. Similar to MG-132, cycloheximide used alone slightly decreased the expression of IκB-α. However, addition of IP6 to cycloheximide treated cells caused a further decrease in IκB-α. Although co-treating PC3 cells with MG-132 and cycloheximide nullified their respective effects on MCL-1 and BIK/NBK expression levels, reduced NOXA and BCL-2 expression remained observable. Interestingly, combined treatment with MG-132 and cycloheximide reduced IκB-α levels more than either treatment used alone independently of IP6.

Discussion

In contrast with studies done using other cell lines (23, 34), the data presented here suggests that, at least in PC3 cells, classical NF-κB subunits p50 and p65 translocate to the nucleus up to 24 hours following treatment with IP6 (Fig. 1a). In parallel, we found that the non-canonical pathway (p100/p52) is largely unaffected by a similar treatment. In parallel, IκB-α protein levels slightly decrease in response to IP6 (Fig. 1a, 2), even though *IκB-α* mRNA increases substantially over 24 hours (Fig. 3b and (25)), beginning as early as 4 hours post-treatment (data not shown). Interestingly, blocking protein production using cycloheximide further decreased the expression of IκB-α protein in response to IP6 (Fig. 6), suggesting that the increase in IκB-α mRNA induced by IP6 may act in an auto-feedback loop to re-establish the levels of IκB-α protein. Although

these results altogether suggest that the NF-κB classical pathway may be triggered in response to treatment of PC3 cells with IP6, inhibiting NF-κB transcriptional activity (assessed by κB-luc assay, Fig. 1b) by transfecting PC3 cells with a non-degradable dominant negative form of IκB-α (29) does not modulate PC3 cell sensitivity to IP6 (Fig. 1c). Hence, our results are at odds with the suggestion that IP6-induced cell death is mediated by inhibition of constitutive NF-κB activity.

Based on our results, a more likely possibility is that IP6 modulates BCL-2 family protein expression in a manner that leads to an increased pro-apoptotic/anti-apoptotic BCL-2 family protein ratio. Indeed, Fig. 2 shows that IP6 induced a gradual shift in the levels of BCL-2 family members at the protein level, wherein pro-apoptotic proteins such as PUMA, NOXA and BIK were up-regulated early on (4 and 8 hours) and where the levels of anti-apoptotic proteins such as MCL-1 and BCL-2 decreased somewhere between 8 and 24 hours following treatment with IP6. Because we had previously observed the up-regulation of *PUMA*, *NOXA* and *BAX* mRNA, we initially hypothesized that this could be mediated by the up regulation of pro-apoptotic genes at the mRNA level. However, we were surprised to find that a transcription inhibitor (actinomycin D, (30)), although effectively blocking IP6-induced up regulation of gene expression (Fig. 3b), did not protect cells from the effects of IP6 (Fig. 3a). As protein expression can be modulated by post-transcriptional events including mRNA export, mRNA translation and proteasome-mediated protein degradation, it remained possible that the ratio of pro-apoptotic to anti-apoptotic proteins could shift in response to IP6 independently of mRNA transcription and of actinomycin D. Indeed, PC3 cells in which protein translation was blocked using cycloheximide were significantly protected from the effects of IP6 (Fig. 3c). At the very least, these data suggest that protein translation is more relevant than mRNA transcription in communicating the observed effects of IP6. With respect to this, it is a likely possibility that the effect of IP6 at least partially involves increased ratios of pro-apoptotic to anti-apoptotic BCL-2 family proteins.

One potential mechanism that could lead to increased protein production independently of transcription is that IP6 stabilizes pro-apoptotic proteins. For example, this could result from the inhibition of specific ubiquitin ligases such as MULE/ARF-BP1 (that targets MCL-1 for degradation) (35) or from direct inhibition of the proteasome. On the other hand, the observed decrease in MCL-1, BCL-2 and IκB-α after longer exposure times of PC3 cells with IP6 argues against both of these possibilities (Fig. 2). Another potential mechanism is that IP6 stimulates ribosomal protein production of pro-apoptotic proteins. Potential candidate targets that could mediate such an effect of IP6 include mTOR and RalA, that has been recently found to regulate the translation of the anti-apoptotic protein $FLIP_S$ (36). Notably, mTOR is well know to be regulated by Akt (37), which in turn is also an upstream activator of NF-κB (7, 10, 11), whose classical p50/p65 subunits translocate to the nucleus in response to IP6 (Fig. 1a). However, activation of mTOR is generally associated to increased expression of cyclin D1 and cell proliferation, not cell death (37). Finally, reports showing that endogenous IP6 plays a role in mRNA export (38, 39) suggest the intriguing possibility is that IP6 increases the cytoplasmic export of existing pools of accumulated pro-apoptotic nuclear mRNA. However, it is unclear whether the nuclear mRNA export machinery is so specific as to allow for the export of pro-apoptotic but not anti-apoptotic BCL-2 family members.

Although the observed effect of cycloheximide on the efficacy of IP6 and IP6/MG-132 co-treatment may involve *de novo* protein synthesis, we cannot exclude the possibility that these observations are linked with reductions in basal pro-apoptotic protein levels resulting from pre-treatment with cycloheximide. Indeed we can observe decreased levels of NOXA and BIK/NBK in response to treatment with cycloheximide alone (Fig. 6, lane 3). On the other hand, treatment with cycloheximide led to a similar decrease in anti-apoptotic MCL-1 (Fig. 6). Surprisingly, we found that MCL-1 levels decreased less in response to treatment with cycloheximide when cells were subsequently treated with IP6 (Fig. 6, compare lane 3 to lane 7). Analogously, IP6-induced expression of PUMA was not fully inhibited in the presence of cycloheximide (Fig. 6 compare lane 4 to lane

7). This observation is interesting in light of reports suggesting that cycloheximide inhibits cytoplasmic ribosomes but not mitochondrial ribosomes, which are of notably different composition (40). Although relatively little is known about nuclear mRNA sorting to the mitochondria (41), mitochondrial import of nuclear tRNAs is better described and is required for mitochondrial protein translation (42, 43). It is tempting to speculate that certain BCL-2 family proteins can be synthesized by mitochondrial ribosomes from imported cytoplasmic mRNAs.

Following from our observations in Fig. 2 and 3, we rationalized that if blocking the production of pro-apoptotic BCL-2 family proteins by treatment with cycloheximide reduced IP6 efficacy, then increasing their levels using proteasome inhibitors may lead to an enhanced effect. Indeed, we found that co-treatment with MG-132 significantly increased the effect of IP6 in at least two AIPCa cell lines (Fig. 4a-b). Our results suggest that this effect is not due to the inhibition of NF-κB transcriptional activity as treatment of PC3 cells with MG-132 increased NF-κB transcription from a κB-luc reporter (Fig. 4b). This is in line with other studies that have reported that in some cells at least, proteasome inhibitors do not inhibit NF-κB (14). In addition, MG-132 did not appear to stabilize the expression of IκB-α, as evidenced by the strong reduction in IκB-α protein observed in cells treated with both cycloheximide and MG-132 with or without IP6 (Fig. 6, lanes 4 and 8). It seems rather unlikely that this is due to a lack of activity on behalf of MG-132, as evidenced by the clear increase in NOXA, BIK/NBK and MCL-1 levels in response to treatment with the proteasome inhibitor. Notably, treatment of PC3 cells with cycloheximide led to decreased levels of these same proteins, suggesting that these may be quickly synthesized and degraded equally rapidly by the proteasome. As MG-132 has been reported to mainly inhibit chymotryptic proteasome activity, this may also suggest that the degradation of IκB-α in prostate cells could be mediated by peptidylglutamyl peptide hydrolyzing, trypsin-like, or even caspase-like proteasome-associated activities (2, 44). In contrast, NOXA, BIK/NBK and MCL-1 may be preferred clients of the chymotryptic active site.

The observation that blocking protein translation using cycloheximide almost completely inhibits MOMP induced by combined IP6 and proteasome-inhibitor treatment strongly points to the involvement of pro-apoptotic BCL-2 family members. In particular, proteins with potentially rapid degradation kinetics such as NOXA and BIK/NBK that are further increased by treatment with IP6 (albeit only slightly) may act in concert to elicit this enhanced effect on mitochondrial depolarization. At the same time, it must be noted that anti-apoptotic MCL-1 also seems to respond, particularly to combined MG-132 and IP6 treatment. With respect to this, it will be interesting to determine whether a MCL-1-targeting siRNA can further enhance the efficacy of combined IP6/proteasome inhibitor treatment. Notably, others were able to increase the potency of Bortezomib using this MCL-1-knockdown approach in melanoma cells (13).

In light of the multitude of pro-apoptotic BCL-2 family members, it is likely that other BCL-2 family members are also up regulated by both IP6 and MG-132, further perturbing the pro-apoptotic/anti-apoptotic ratio. It is equally possible that a subset of pro-apoptotic proteins is specifically up regulated by IP6, while another complementary subset is up regulated by MG-132. This could lead to enhanced cytotoxicity according to a recently proposed hierarchical BCL-2 family model of BAX/BAK activation. In this model, some BH3-only proteins such as NOXA, BAD, BMF and BIK/NBK may act up-stream of "activator-only" pro-apoptotic proteins such as BID, BIM, and PUMA, by releasing them from the inhibitory clutches of anti-apoptotic BCL-2/BCL-XL and MCL-1 (19). In this context, the combined up regulation of NOXA and BIK/NBK by MG-132 may complement the up-regulation of activator-only proteins following challenge with IP6. Although PUMA was a potential candidate, its expression also appears to be up regulated by MG-132 (Fig. 6 lane 2). In addition, our attempts to prevent the enhanced cytotoxic effect of combined IP6 and MG-132 treatment by shRNA-mediated down-regulation of PUMA have proved unsuccessful so far (data not shown). Hence, we suspect that other pro-apoptotic "activator-only" proteins may be up regulated by IP6 but not by MG-132, or that alternately IP6 induces post-

translational modifications that activate pro-apoptotic proteins up regulated by MG-132. We are currently investigating these possibilities.

Nonetheless, the enhanced effect of IP6 was seen when combined with at least two proteasome inhibitors. This was most clearly visible when MOMP was assessed in individual PC3 cells using JC-1 dye (Fig. 5a-c). In addition to the observation that combining IP6 with MG-132 leads to an enhanced effect in at least two AIPCa cell lines, these results suggest that IP6 may be useful in combination with proteasome inhibitor drugs for treatment of AIPCa. This possibility is particularly exciting since proteasome inhibitors are already undergoing pre-clinical trials for AIPCa (3-5) and since previous studies have also shown that IP6 can exhibit anti-cancer activity in mice when administered as a dietary supplement in drinking water (45).

Conclusion

In conclusion, we find that IP6 triggers an NF-κB response in PC3 cells but that this response does not likely play a major role in either preventing or promoting the cytotoxic effects of IP6. We propose that the up-regulation of pro-apoptotic BCL-2 family members at the protein level is likely involved in skewing the pro-apoptotic/anti-apoptotic protein ratio and in mediating the cytotoxic effects of IP6. Finally, we conclude that proteasome inhibitors enhance the effect of IP6 on mitochondrial depolarization, potentially involving multiple pro-apoptotic BCL-2 family members. Altogether, our results suggest that the pre-clinical evaluation of proteasome inhibitors in combination with IP6 is warranted and could provide an alternative for the treatment of AIPCa.

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References

1. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M. J. Cancer statistics, 2007. CA Cancer J Clin, *57:* 43-66, 2007.

2. Lee, D. H. and Goldberg, A. L. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol, *8:* 397-403, 1998.

3. Papandreou, C. N., Daliani, D. D., Nix, D., Yang, H., Madden, T., Wang, X., Pien, C. S., Millikan, R. E., Tu, S. M., Pagliaro, L., Kim, J., Adams, J., Elliott, P., Esseltine, D., Petrusich, A., Dieringer, P., Perez, C., and Logothetis, C. J. Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer. J Clin Oncol, *22:* 2108-2121, 2004.

4. Papandreou, C. N. and Logothetis, C. J. Bortezomib as a potential treatment for prostate cancer. Cancer Res, *64:* 5036-5043, 2004.

5. Price, N. and Dreicer, R. Phase I/II trial of bortezomib plus docetaxel in patients with advanced androgen-independent prostate cancer. Clin Prostate Cancer, *3:* 141-143, 2004.

6. Zavrski, I., Jakob, C., Kaiser, M., Fleissner, C., Heider, U., and Sezer, O. Molecular and clinical aspects of proteasome inhibition in the treatment of cancer. Recent Results Cancer Res, *176:* 165-176, 2007.

7. Chen, L. F. and Greene, W. C. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol, *5:* 392-401, 2004.

8. Buss, H., Dorrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. J Biol Chem, *279:* 55633-55643, 2004.

9. Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science, *298:* 1241-1245, 2002.

10. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer, *2:* 301-310, 2002.

11. Karin, M. and Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol, *18:* 621-663, 2000.

12. Marshansky, V., Wang, X., Bertrand, R., Luo, H., Duguid, W., Chinnadurai, G., Kanaan, N., Vu, M. D., and Wu, J. Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. J Immunol, *166:* 3130-3142, 2001.

13. Qin, J. Z., Xin, H., Sitailo, L. A., Denning, M. F., and Nickoloff, B. J. Enhanced Killing of Melanoma Cells by Simultaneously Targeting Mcl-1 and NOXA. Cancer Res, *66:* 9636-9645, 2006.

14. Zhu, H., Zhang, L., Dong, F., Guo, W., Wu, S., Teraishi, F., Davis, J. J., Chiao, P. J., and Fang, B. Bik/NBK accumulation correlates with apoptosis-induction by bortezomib (PS-341, Velcade) and other proteasome inhibitors. Oncogene, *24:* 4993-4999, 2005.

15. Concannon, C. G., Koehler, B. F., Reimertz, C., Murphy, B. M., Bonner, C., Thurow, N., Ward, M. W., Villunger, A., Strasser, A., Kogel, D., and Prehn, J. H. Apoptosis induced by proteasome inhibition in cancer cells: predominant role of the p53/PUMA pathway. Oncogene, *26:* 1681-1692, 2007.

16. Nikrad, M., Johnson, T., Puthalalath, H., Coultas, L., Adams, J., and Kraft, A. S. The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim. Mol Cancer Ther, *4:* 443-449, 2005.

17. Kroemer, G., Galluzzi, L., and Brenner, C. Mitochondrial membrane permeabilization in cell death. Physiol Rev, *87:* 99-163, 2007.

18. Willis, S. N. and Adams, J. M. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol, *17:* 617-625, 2005.

19. Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J., and Cheng, E. H. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol, *8:* 1348-1358, 2006.

20. Vucenik, I. and Shamsuddin, A. M. Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic. J Nutr, *133:* 3778S-3784S, 2003.

21. Fox, C. H. and Eberl, M. Phytic acid (IP6), novel broad spectrum anti-neoplastic agent: a systematic review. Complement Ther Med, *10:* 229-234, 2002.

22. Zi, X., Singh, R. P., and Agarwal, R. Impairment of erbB1 receptor and fluid-phase endocytosis and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells. Carcinogenesis, *21:* 2225-2235, 2000.

23. Agarwal, C., Dhanalakshmi, S., Singh, R. P., and Agarwal, R. Inositol hexaphosphate inhibits constitutive activation of NF- kappa B in androgen-independent human prostate carcinoma DU145 cells. Anticancer Res, *23:* 3855-3861, 2003.

24. Singh, R. P., Agarwal, C., and Agarwal, R. Inositol hexaphosphate inhibits growth, and induces G1 arrest and apoptotic death of prostate carcinoma DU145 cells: modulation of CDKI-CDK-cyclin and pRb-related protein-E2F complexes. Carcinogenesis, *24:* 555-563, 2003.

25. Diallo, J. S., Peant, B., Lessard, L., Delvoye, N., Le Page, C., Mes-Masson, A. M., and Saad, F. An androgen-independent androgen receptor

function protects from inositol hexakisphosphate toxicity in the PC3/PC3(AR) prostate cancer cell lines. Prostate, *66:* 1245-1256, 2006.

26. Agarwal, C., Dhanalakshmi, S., Singh, R. P., and Agarwal, R. Inositol hexaphosphate inhibits growth and induces G1 arrest and apoptotic death of androgen-dependent human prostate carcinoma LNCaP cells. Neoplasia, *6:* 646-659, 2004.

27. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res, *29:* e45, 2001.

28. Sun, S. C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science, *259:* 1912-1915, 1993.

29. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science, *267:* 1485-1488, 1995.

30. Yamamoto, N., Mueller-Lantzsch, N., and zur Hausen, H. Effect of actinomycin D and cycloheximide on Epstein-Barr virus early antigen induction in lymphoblastoid cells. J Gen Virol, *51:* 255-261, 1980.

31. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell, *78:* 773-785, 1994.

32. Salvioli, S., Ardizzoni, A., Franceschi, C., and Cossarizza, A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett, *411:* 77-82, 1997.

33. Schow, S. R. and Joly, A. N-acetyl-leucinyl-leucinyl-norleucinal inhibits lipopolysaccharide-induced NF-kappaB activation and prevents TNF and IL-6 synthesis in vivo. Cell Immunol, *175:* 199-202, 1997.

34. Ferry, S., Matsuda, M., Yoshida, H., and Hirata, M. Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NFkappaB-mediated cell survival pathway. Carcinogenesis, *23:* 2031-2041, 2002.

35. Zhong, Q., Gao, W., Du, F., and Wang, X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell, *121:* 1085-1095, 2005.

36. Panner, A., Nakamura, J. L., Parsa, A. T., Rodriguez-Viciana, P., Berger, M. S., Stokoe, D., and Pieper, R. O. mTOR-independent translational control of the extrinsic cell death pathway by RalA. Mol Cell Biol, *26:* 7345-7357, 2006.

37. Dutcher, J. P. Mammalian target of rapamycin inhibition. Clin Cancer Res, *10:* 6382S-6387S, 2004.

38. Weirich, C. S., Erzberger, J. P., Flick, J. S., Berger, J. M., Thorner, J., and Weis, K. Activation of the DExD/H-box protein Dbp5 by the nuclear-pore protein Gle1 and its coactivator InsP6 is required for mRNA export. Nat Cell Biol, *8:* 668-676, 2006.

39. Alcazar-Roman, A. R., Tran, E. J., Guo, S., and Wente, S. R. Inositol hexakisphosphate and Gle1 activate the DEAD-box protein Dbp5 for nuclear mRNA export. Nat Cell Biol, *8:* 711-716, 2006.

40. Bruce, I. J. and Kerry, R. The effect of chloramphenicol and cycloheximide on platelet aggregation and protein synthesis. Biochem Pharmacol, *36:* 1769-1773, 1987.

41. Sylvestre, J., Margeot, A., Jacq, C., Dujardin, G., and Corral-Debrinski, M. The role of the 3' untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. Mol Biol Cell, *14:* 3848-3856, 2003.

42. Kamenski, P., Kolesnikova, O., Jubenot, V., Entelis, N., Krasheninnikov, I. A., Martin, R. P., and Tarassov, I. Evidence for an adaptation

mechanism of mitochondrial translation via tRNA import from the cytosol. Mol Cell, *26:* 625-637, 2007.

43. Verner, K. Co-translational protein import into mitochondria: an alternative view. Trends Biochem Sci, *18:* 366-371, 1993.

44. Groll, M., Heinemeyer, W., Jager, S., Ullrich, T., Bochtler, M., Wolf, D. H., and Huber, R. The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study. Proc Natl Acad Sci U S A, *96:* 10976-10983, 1999.

45. Singh, R. P., Sharma, G., Mallikarjuna, G. U., Dhanalakshmi, S., Agarwal, C., and Agarwal, R. In vivo suppression of hormone-refractory prostate cancer growth by inositol hexaphosphate: induction of insulin-like growth factor binding protein-3 and inhibition of vascular endothelial growth factor. Clin Cancer Res, *10:* 244-250, 2004.

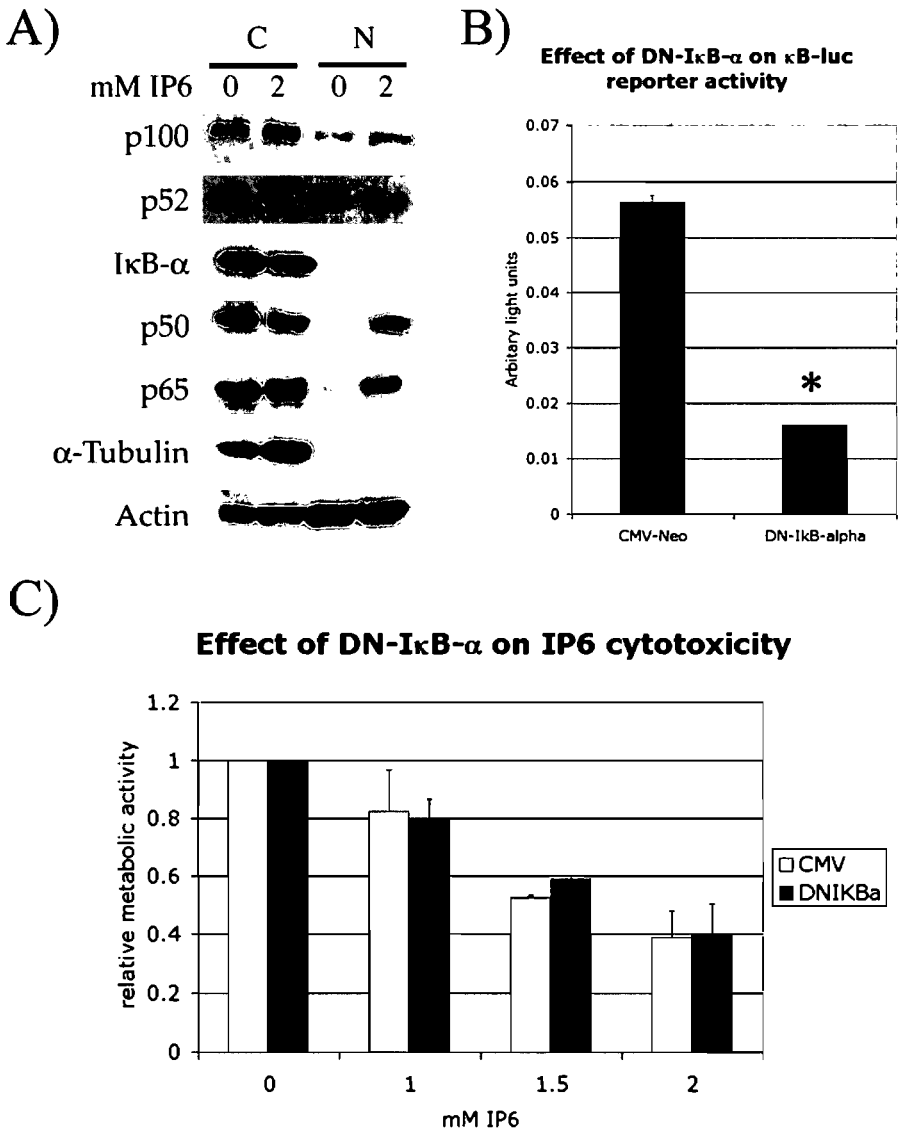

Figure 1.NF-κB in the response to IP6

Figure 1. A) IP6 induces canonical NF-κB subunit nuclear translocation. Nuclear (N) and cytoplasmic (C) extracts of PC3 cells treated with vehicle (0 mM) or IP6 (2 mM) for 24 hours, were probed by Western blot to assess the expression and subcellular localization of p100, p52, IκB-α, p50, and p65. α-TUBULIN was used as a control for nuclear extract purity. β-ACTIN was used as a loading control. B) A non-degradable dominant-negative of IκB-α inhibits NF-κB transcriptional activity. DN-IκB-α or pCMVNeo control plasmids were co-transfected with κB-luc/Renilla luciferase in PC3 cells. κB-luc activity was measured after 72 hours and normalized according to renilla and basal tkGL3 activity (see Materials and Methods). Data represents average of 2 independent experiments done in 4-8 replicates each. *p-value <0.05. C) Inhibition of NF-κB transcriptional activity does not modulate the efficacy of IP6. PC3 cells transiently transfected (48h) with pCMVNeo and DN-IκB-α cells were plated at a density of 20 000 cells per well and treated with increasing doses of IP6. Metabolic activity was measured using WST-1 reagent and relative metabolic activity was normalized according to vehicle-treated pCMVNeo or DN-IκB-α transfected cells (see Materials and Methods). Data represents average of 2 independent experiments done in 3-6 replicates each. Error bars represent the standard error.

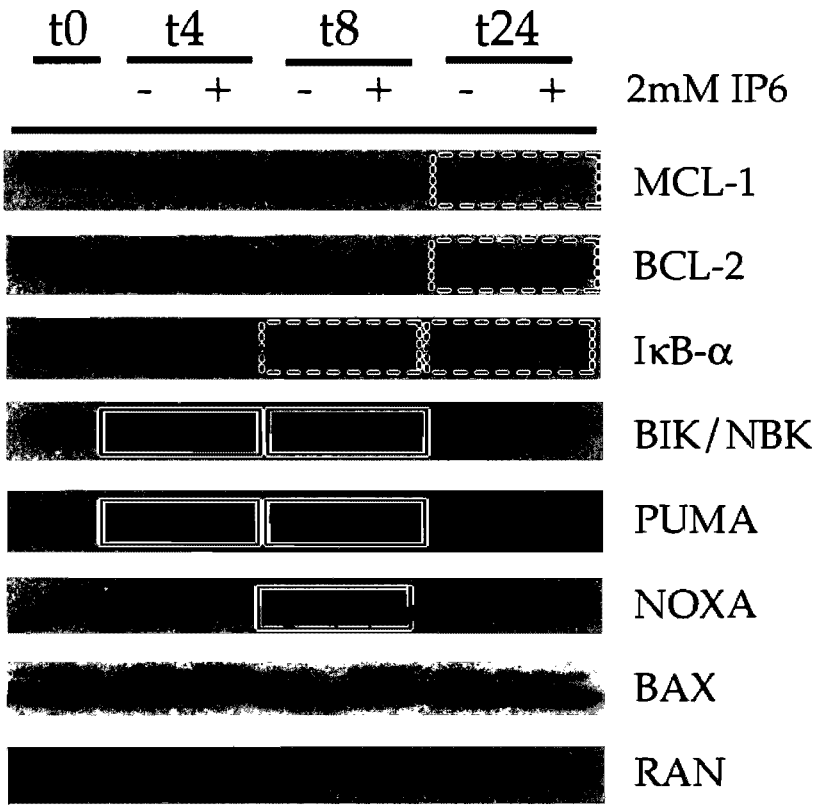

Figure 2. IP6 induces temporal changes in the levels of BCL-2 family proteins

Figure 2. IP6 modulates the expression of BCL-2 family proteins. PC3 cells were treated with 2mM IP6 or vehicle and whole cell extracts were prepared from cells harvested upon treatment (t0) and after 4 (t4) and 8 (t8) hours of additional treatment with IP6 (+) or vehicle (-). Western blotting was used to probe extracts for MCL-1, BCL-2, IκB-α, BIK/NBK, PUMA, NOXA, and BAX expression. RAN expression was used as a loading control. Solid boxes signal increased protein levels in treated cells as compared to time-matched control cells whereas dashed boxes indicate decreased protein levels relative to time-matched controls. Blot was representative of 2 independent experiments each done in duplicate.

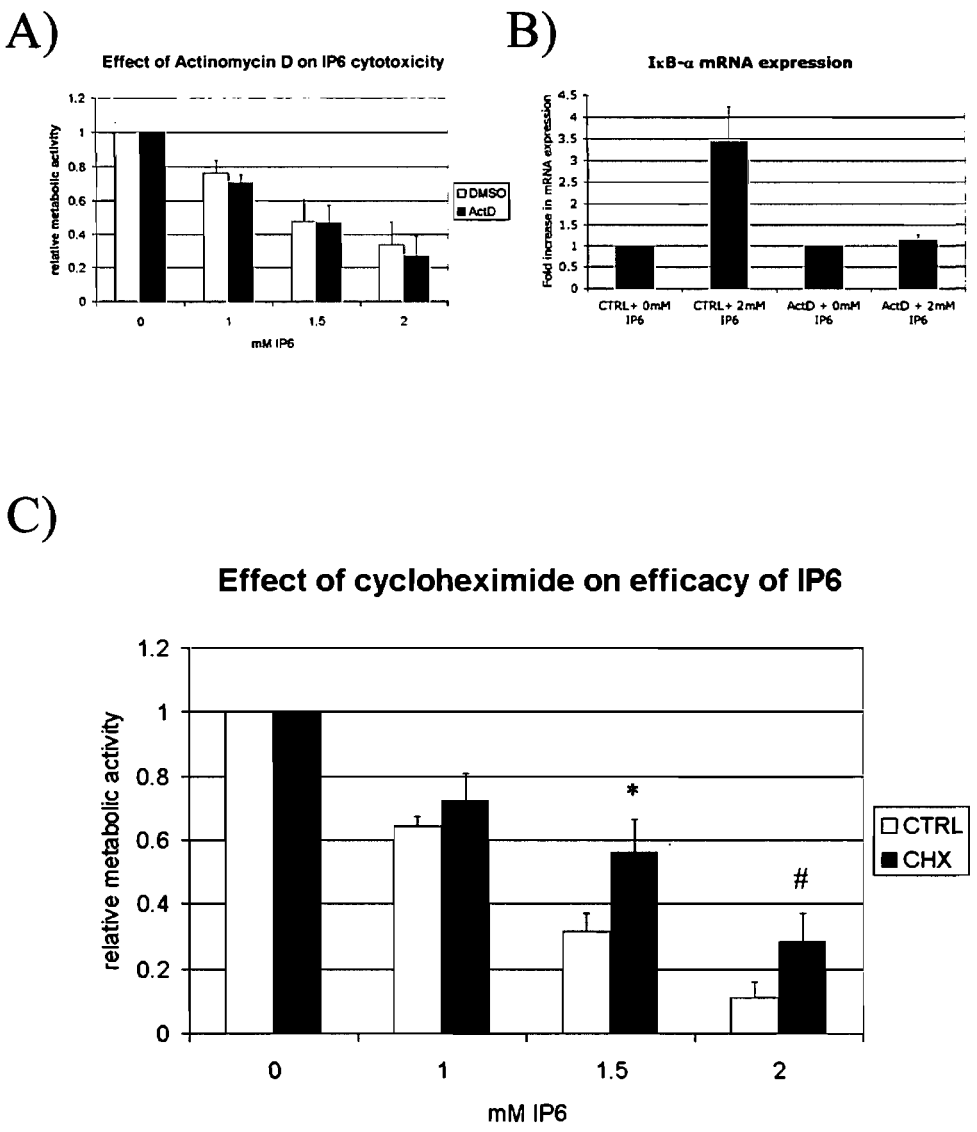

Figure 3. The effect of IP6 requires protein translation but not mRNA transcription

Figure 3. A) A transcription inhibitor does not protect PC3 cells from the effects of IP6. Actinomycin D (1 μg/ml) and DMSO pre-treated PC3 cells (density of 20 000 cells per well) were treated with increasing doses of IP6 for 24 hours. Metabolic activity was measured after 24 hours (plus 4-hour pre-treatment) using WST-1 and the data was normalized according to DMSO or actinomycin D pre-treated cells not treated with IP6. Data represents average of 5 independent experiments each done in 3-6 replicates. No significant difference was observed between actinomycin D and DMSO treated cells. B) Actinomycin D inhibits IP6-induced up regulation of IκB-α mRNA. PC3 cells were pre-treated for 4 hours with 1 μg/ml actinomycin D or DMSO (CTRL) then treated with 2mM IP6 or vehicle (0mM). cDNA were synthesized from RNA extracted 24 hours later and real-time PCR was used to measure the fold change in IκB-α mRNA expression. Data represents an average of 2 independent experiments done in duplicate. C) An inhibitor of protein translation protects PC3 cells from the effects of IP6. As in B, cycloheximide (50 μg/ml) and ethanol pre-treated PC3 cells were treated with increasing doses of IP6 for 24 hours and metabolic activity was measured using WST-1. Data represents an average of 3 independent experiments done in 3-6 replicates. Error bars represent the standard error. *p-value <0.05, #p-value=0.06.

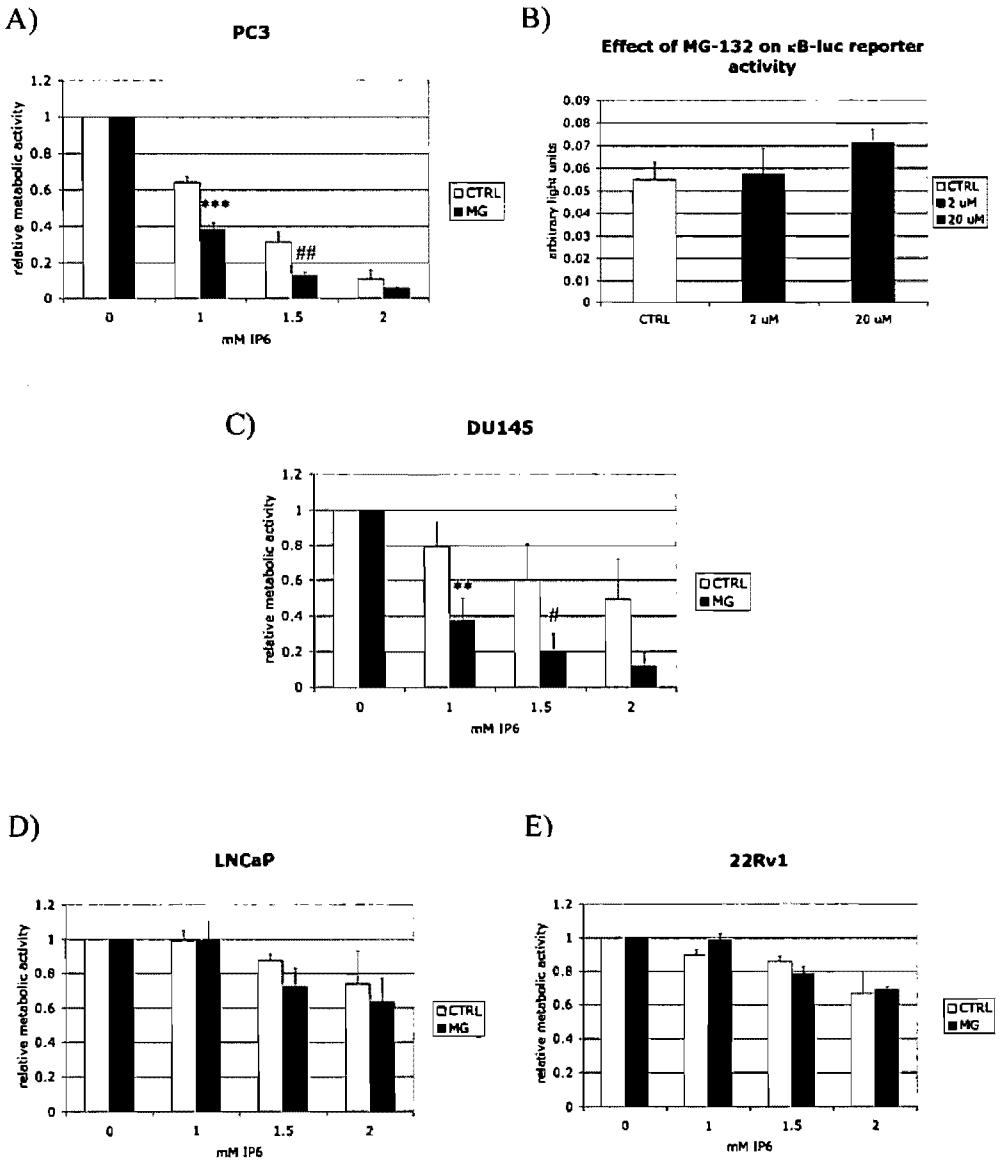

Figure 4. A proteasome inhibitor sensitizes AIPCa cells to the effect of IP6

Figure 4. The proteasome inhibitor MG-132 sensitizes AIPCa cells to the effects of IP6. In A) (PC3), C) (DU145), D) (LNCaP) and E) (22Rv1), cells were plated at 20 000 cells per well and pre-treated for 4 hours with MG-132 (or ethanol control) prior to addition of IP6 at increasing concentrations. Metabolic activity was measured 24 hours later using WST-1. For each cell line, relative metabolic activity was calculated according to the WST-1 output MG-132-treated or control cells treated with vehicle (0 mM IP6). Data represent average of 3 independent experiments done in 3-6 replicates each. ***p-value<0.005, **p-value<0.01, #p-value=0.08, ##p=0.09 B) As described in Fig. 1c, κB-luc reporter and control-transfected cells were treated with 2 or 20 μM MG-132 or vehicle (CTRL) and assayed for relative luciferase activity. Data represents average of 2 independent experiments done in 4-8 replicates each. Error bars represent the standard error.

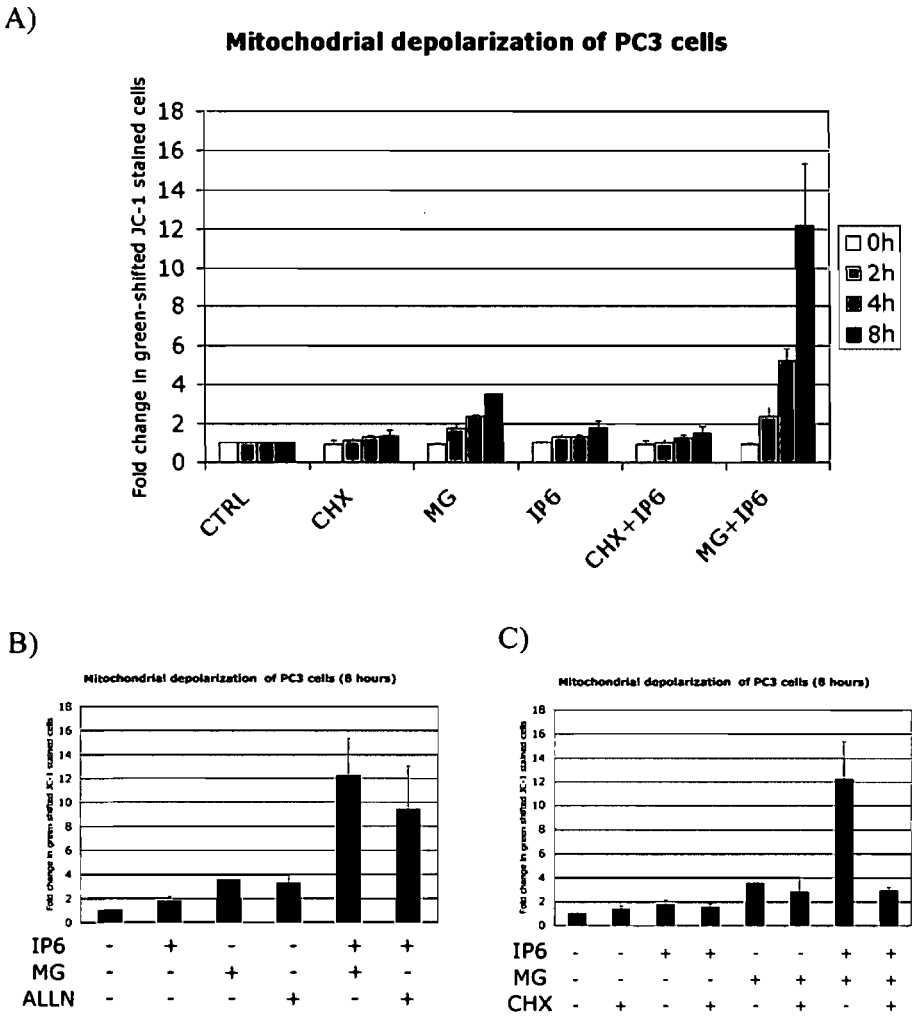

Figure 5. IP6 and proteasome inhibitors present an enhanced effect on mitochondrial depolarization that requires protein translation.

Figure 5. IP6 and proteasome inhibitors present an enhanced effect on mitochondrial depolarization. A) PC3 cells pre–treated with 50 μg/ml cycloheximide (CHX), 20 μM MG-132 (MG) or control ethanol and were stained with JC-1 dye 0, 2, 4, and 8 hours following treatment with 2mM IP6 (or vehicle). Cells were trypsinized and sorted by FACS and the percentage of green-shifted JC-1 cells was assessed. Data was then normalized to vehicle-treated control for each time point. Data represents an average of 3 independent experiments. B) PC3 cells were pre-treated with 20 μM MG-132, 10 μM ALLN, or DMSO prior to addition of 2mM IP6 (or control). Eight hours later, green-shifted JC-1 stained cells were counted by FACS and data was normalized according to vehicle treated controls. Data represents average of 2-3 experiments. All error bars represent the standard error. C) PC3 cells were pre-treated with 20 μM MG-132, 50 μg/ml cycloheximide, or a combination of MG-132 and cycloheximide or control ethanol prior to addition of 2mM IP6 (or vehicle). 8 hours later, green-shifted JC-1 stained cells were counted by FACS and data was normalized according to vehicle treated controls. Data represents average of 2-3 experiments.

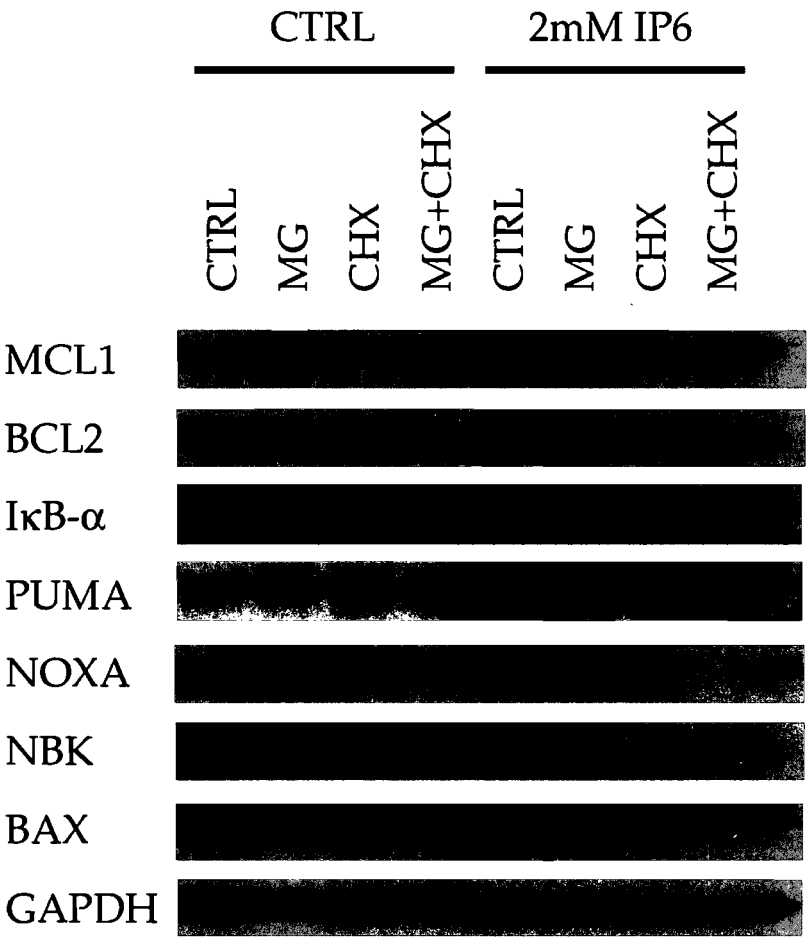

Figure 6. IP6 and MG-132 skew pro-apoptotic to anti-apoptotic protein ratios by altering the levels of BCL-2 family proteins

Figure 6. Modulation of BCL-2 family protein expression in PC3 cells in response to IP6. MG-132 (MG), and cycloheximide (CHX). PC3 cells were pre-treated with 20 μM MG-132, 50 μg/ml cycloheximide or both. Four hours later, cells were treated with 2mM IP6 (or control) for an additional 4 hours. Whole cell extracts were prepared and probed for MCL-1, BCL-2, IκB-α, PUMA, NOXA, BIK/NBK and BAX by Western blot. GAPDH was used as a loading control. The presented blots were representative of 3 independent experiments each done in duplicate.

CHAPTER VI

6. Discussion

6.1. AR expression and the sensitivity to IP6

In chapter 2, we explored the effect of IP6 on four commonly used PCa cell lines and observed that the efficacy of IP6 correlated with AR expression. In this study we found that AR-negative DU145 and PC3 cells were more sensitive to IP6 than AR-positive LNCaP and 22Rv1 cells. In light of these observations, we went on to determine whether the sensitivity of IP6 could be modulated by supplementation with androgens or steroid depletion. Although our results suggested that the efficacy of IP6 was not modulated by androgens, we observed that PC3 cells stably transfected with the AR were more resistant to the effects of IP6 than wild-type PC3 cells. Furthermore, an siRNA targeted against the AR could sensitize PC3(AR) cells to the effects of IP6 although this was insufficient to sensitize LNCaP or 22Rv1 cells. In addition, we found that in PC3 cells, treatment with IP6 led to an increase in caspase-3 like activity as well as to an increase in DNA fragmentation. The mRNA expression of a subset of NF-κB-responsive genes (*IκB-α, IRF-2*) and of genes encoding for pro-apoptotic proteins (*PUMA, NOXA and BAX*) was also found to increase in response to treatment with IP6. All of these events were significantly reduced in PC3(AR) cells. At least in the context of PC3/PC3(AR) cells, this suggested the implication of an androgen-independent AR-associated function implicated in the resistance to IP6 cytotoxicity.

The nature of the androgen-independent AR-associated function that led to decreased IP6 efficacy in PC3(AR) cells according to WST-1 metabolic assays, fluorigenic caspase-3 substrate assays, DNA fragmentation (by filter elution) and gene expression profiling remains obscure. Based on the observation that IP6 led to substantial up regulation of the p50/p65-responsive gene *IκB-α* [590]) but only minimally affected the expression of p52-responsive *CyclinD1* and *BCL-2*, [143, 591]), we initially proposed that IP6 could preferentially activate the classical NF-κB p50/p65 pathway in PC3 cells (Chapter II, Fig. 5). We also hypothesized that

through a putative interaction with p65/RELA [370] stable expression of the AR could potentially inhibit classical NF-κB activity and protect from the effects of IP6 in light of the observation that NF-κB can be pro-apoptotic in some circumstances [592, 593]. Although the results obtained in chapter V (Chapter V, Fig 1a) suggest that our initial hypothesis on the activation of the classical versus non-classical NF-κB pathway may have been correct, the observation that reducing NF-κB transcriptional activity does not modulate sensitivity to IP6 in PC3 cells (Chapter V Fig 1b-c) argues against our second proposition.

In light of a similar effect of stable AR expression on the up regulation of pro-apoptotic genes in response to treatment with IP6, particularly of *PUMA*, *NOXA* and *BAX*, we considered the possibility that the up-regulation of pro-apoptotic mRNA could play a role in the observed effect of IP6. We also proposed that given that PC3 cells harbor a p53 mutation leading to a premature stop codon and a p53-null phenotype, potential alternative mediators of *PUMA* and *NOXA* up-regulation (initially described as p53-responsive genes) could be E2Fs. As the transcriptional activity of E2Fs is regulated by pRb-family proteins, and since the AR has been reported to interact with pRb in an androgen-independent fashion [357], such a role of E2Fs could have explained our observations. At least two of these hypotheses are likely refutable. First, it appears from preliminary experiments using a dominant-negative E2F construct that blocking E2F transcription does not modulate the efficacy of IP6 (unpublished results). Second, experiments using actinomycin D done in chapter V (Chapter V, Fig 3a-b) suggest that inhibiting the transcriptional response induced by IP6 does not modulate the efficacy of IP6. Although these results do not exclude a role of E2F transcription factors in mediating PUMA and NOXA expression and do not eliminate the possibility of an androgen-independent interaction of pRb with the AR, they certainly argue against a primary role of E2F transcription factors in mediating the response to IP6.

Although we were able to observe DNA fragmentation in response to treatment with IP6, it must be noted that this hallmark of apoptosis is not exclusive and can result from other modes of cell death, including necrosis (see section). In addition, IP6-induced cell death may not present all of the characteristic hallmarks of apoptosis. One study found that treatment of DU145 cells with 2mM IP6 induced an increase of only roughly 2% in cell surface exposed membrane phosphatidyl serine as measured by Annexin V combined with fluorescence activated cell sorting. In parallel, the cleavage of PARP and caspase-3 were evident following a similar treatment [585]. Furthermore, we have found that a pan-caspase inhibitor ZVAD-fmk, while effectively blocking caspase-3 activity, does not modulate the effects of IP6 on the metabolic activity of PC3 cells (unpublished results). Similarly, ZVAD was found to have no impact on the efficacy of IP6 in mouse prostate cancer cells [594]. Hence, it remains possible that at least a proportion of cells die by necrosis or another form of caspase-independent death.

With respect to this, some studies suggest that likely depending on the mode of death, the cellular response to drug treatments as measured by metabolic assays such as WST-1 may appear with slower or faster kinetics as compared to biochemical assays looking more specifically at hallmarks of apoptosis or necrosis respectively [595, 596]. By comparing the results obtained using metabolic activity (WST-1) and apoptosis markers (such as caspase-3 activation and DNA fragmentation) as endpoints for assessing IP6 efficacy, we noted that the effect of IP6 appeared greatest at the metabolic level. For example, whereas a 24 hour treatment of PC3 cells with 2mM IP6 reduced metabolic activity by approximately 80-90% (Chapter II, Fig 1a, 2b, 3a-b) we only observed a 4-fold increase in caspase-3 activity in response to a similar treatment with IP6 (Chapter II, Fig. 3f). This increase is modest when we consider that for a similar effect of the DNA damaging agent camptothecin on metabolic activity in 22Rv1 cells, a nearly 120-fold increase in caspase-3 activity is observed (unpublished preliminary data). Similarly, we detected an increase of roughly 15% in fragmented DNA 24-hours following treatment with 2mM IP6 (Chapter II, Fig.

3g). At 48 hours, 30% of the DNA was fragmented which is still far from the 80% reduction in metabolic activity typically observed after only 24 hours of treatment with IP6. Hence, these observations support the possibility that IP6 induces caspase-independent modes of cell death in a certain proportion of cells.

Interestingly, cells having initiated the apoptotic program can undergo necrosis or caspase-independent death when cellular stores of ATP are rapidly depleted [197]. Importantly, WST-1 measures the activity of NADH oxidoreductases, indicating proper electron transfer chain activity and ATP production [597]. As mentioned in section 1.2.3.1.1, the electron transfer chain requires the activity of several cytochrome complexes. Interestingly, cytochrome proteins contain heme prosthetic groups and are very closely related to hemoglobin, a protein that carries oxygen in the blood [598, 599]. Notably, IP6 is well known to tightly bind hemoglobin and release heme-oxygen complexes [600]. As such it is not inconceivable that IP6 interferes with the electron transfer chain present within the mitochondrial inner membrane, thus reducing metabolic activity and consequently WST-1 output. In this context, it is tempting to speculate that the known interaction of the AR with mitochondrial electron chain transfer components such as COXVb [362] could explain the protective effect of the AR.

One intriguing question is why siRNA-mediated depletion of the AR in cell lines that endogenously express it, such as LNCaP and 22Rv1 cells, does not sensitize these cells to the effects of IP6. This observation argues against a direct role of the AR in mediating the resistance to IP6 at least in these cells. One possibility is that the effect of the AR is indirect and that it involves a secondary AR transcriptional target. As such this target would need to be extremely stable so as to not decrease in expression following a transient knockdown of the AR in these cells (chapter II figure 4a). At the same time, we would expect a similar result in PC3(AR) cells if this were the case. On the other hand, it may be that proteins that regulate the degradation of this hypothetical "IP6-resistance protein",

such as ubiquitin ligases for example, are absent in LNCaP and 22Rv1 but are active in PC3 and DU145 cells.

Another possibility is that AIPCa cells that have evolved to survive in complete absence of AR expression (or that originate from AR-negative prostate cells such as neuro-endocrine or basal cells) are fundamentally different from PCa cells that express the AR. This notion has been evoked by others to explain why androgens reduce cellular proliferation and induce apoptosis in PC3 cells stably transfected with the AR while a similar treatment increases proliferation in AR positive cells [601]. As such, it is possible that the molecular signature shared by AR-negative DU145 and PC3 cells is linked to IP6 sensitivity and that the expression of the AR interferes with components specific to this "AR-negative signature" in PC3 cells, leading to IP6 resistance.

Although the results are somewhat controversial, some studies suggest that a similar androgen-independent AR-linked phenomenon may be observed when looking at the response to DNA-damaging agents. One study suggests that cytoplasmic androgen-bound AR leads to the activation of MAPK and to the resistance to the DNA-damaging agent etoposide in the context of AR-negative HeLa and Osteoblast cells [396]. On the other hand, another study reported that androgens sensitizes to the effects of DNA-damaging agents in AR-positive prostate cells. Interestingly, this study showed that PC3(AR) cells were sensitized to DNA-damaging agents even in absence of androgens. This phenomenon was suggested to be mediated by the androgen-dependent up regulation of NOXA [602]. However, our observations are somewhat at odds with a role of NOXA since the data presented in chapter III (Chapter III, Fig 1) suggest that NOXA expression is in fact higher in AR-negative AIPCa cells. In addition, we did not observe increased NOXA expression in the PC3(AR) cell line used in chapter II (unpublished results).

Finally, one interesting possibility is brought to light by the recent finding that in PC3 cells, the AR inhibits EGFR signaling in an androgen-independent manner by interfering with receptor internalization through the caveolin-1/clathrin pathway [397]. As mentioned in section 1.4.4.2.2, one study suggests that treatment of DU145 cells with IP6 also leads to inhibition of EGFR signaling through receptor internalization, among others through decreased binding of EGFR to the AP-2 clathrin protein [537]. As such, the relative effect of IP6 may be decreased in a context where the AR already exerts an analogous function. On the other hand, we would once again expect that AR down-regulation would have a similar effect in PC3(AR), LNCaP and 22Rv1 cells. This is of course notwithstanding the possibility that AR-negative and AR-positive exhibit differences in ErbB family member expression and sub-cellular localization, or in the rates of receptor endocytosis. In relation to this, we have recently shown that ErbB3 translocates to the nucleus of PCa cells [603]. In this study, we found that LNCaP and 22Rv1 cells exhibited more nuclear ErbB3 than PC3 or DU145 cells. In light of this, it will be interesting to determine whether sub-cellular localization of ErbB family members can influence the efficacy of IP6.

6.2. NOXA and PUMA as molecular targets and prognostic markers for prostate cancer

Following our initial observations made in chapter II, we hypothesized that pro-apoptotic BCL-2 family proteins including NOXA, PUMA and BAX may play a role in the mediating the effects of IP6. Although the expression of BAX had been well studied in the context of the prostate, PUMA and NOXA expression had not yet been characterized in prostate tissues. As such, we set out to characterize the expression of NOXA and PUMA in prostate tissues and to assess their relationship to biochemical recurrence (BCR) in chapter III. Using immunohistochemical methods and a novel color scale method, we assessed NOXA and PUMA expression in tissue micro-arrays encompassing tissues from autopsied cancer-free patients, radical prostatectomy specimens from hormone-sensitive PCa patients (including non-neoplasic, PIN, and cancerous regions), as

well as TURP specimens collected from AIPCa patients. We observed that NOXA and PUMA were expressed in all prostate tissues analyzed and that both were frequently up-regulated in PCa when comparing non-neoplasic to cancerous tissues obtained from the same radical prostatectomy specimens (68% for NOXA and 70% for PUMA). While on average PUMA expression appeared to attain peak levels in hormone-sensitive PCa samples, NOXA expression was at its highest in AIPCa tissues. In contrast with PUMA, we found that high NOXA expression was associated to increased risk of BCR. Several survival tree models were subsequently generated using NOXA and PUMA staining intensity, the Gleason Score, surgical margin status, pathological stage and pre-operative PSA, as input parameters. These were then ranked according to the predictive accuracy. From these analyses, we found a recurrent structure within the top ranking survival trees, where patient BCR risk was first stratified according to surgical margin status then according to NOXA expression within patients with negative surgical margins. On the basis as of this and other practical considerations, we proposed that a model including surgical margin status, NOXA expression, and pre-operative PSA may warrant further validation.

From the data presented in chapter III, one can argue that NOXA and PUMA could be considered as potential targets for PCa treatment. Both proteins are expressed in all PCa specimens encountered and are frequently over-expressed in cancerous relative to adjacent non-neoplasic tissues. Average PUMA and NOXA staining intensities are at their lowest in normal tissues from cancer-free patients and at their highest in cancerous tissues (Chapter III, Fig 3a-b). In the case of NOXA, levels were at their highest in AIPCa patients. Whether PUMA and NOXA can be further activated beyond mere protein up-regulation remains somewhat of a hypothetical concept; however, if this were to occur, we can predict therapies that increase the activity of NOXA and PUMA may hold some therapeutic benefit for PCa and AIPCa patients. Indirectly, this finding also argues for the development of siRNA strategies targeting anti-apoptotic BCL-2 family members, such as Oblimersen that targets BCL-2, for use against PCa and

AIPCa [510]. As such, we would predict that antisense drugs such as Oblimersen would have a greater effect in cells where the expression levels of pro-apoptotic BCL-2 family members are already elevated.

When looking at the ability of NOXA and PUMA to predict BCR in patients having undergone radical prostatectomy, we found that NOXA expression was adversely predictive of BCR, but that PUMA on its own was not predictive of BCR (Chapter III, Fig 4a-b). Although it is quite possible that PUMA is not well suited as a prognostic marker, it must be noted that the antibody used in this study may not be specific for only one isoform of PUMA. Although we found that, to various extents, all of the PCa cell lines expressed pro-apoptotic PUMA-α (22 kDA band; Chapter III, Fig.1), the better known BH3-containing pro-apoptotic isoform (generally referred to as PUMA), we cannot exclude the possibility that other isoforms are expressed in prostate tissues. Notably, the expression of isoforms that don't contain a BH3 domain and whose functions are poorly understood such as PUMA-δ and γ, could confound the conclusions drawn with respect to the prognostic value of *bone fide* pro-apoptotic PUMA, which is primarily associated to both α and β isoforms [117]. It will be interesting to determine whether the different PUMA isoforms could hold different prognostic information. However, to this effect, the availability of antibodies that are truly specific for each isoform may pose the greatest challenge.

As discussed in chapter III, the observation of increased NOXA and PUMA expression in PCa in addition to the finding that high expression of NOXA is associated to a worse prognostic is somewhat counter-intuitive. We emitted the hypothesis that, counter to increased expression of pro-apoptotic proteins, there may be a parallel increase in cell proliferation, leading to increased tissue turnover. Recently, by comparing our results with those of another mutli-marker study done on the same tissue micro-arrays (Gannon PO et al., Clin Cancer Res, submitted), we observed that within the sub-cohort of PCa patients having undergone radical prostatectomy, patients that exhibited high NOXA

expression also exhibited an increased frequency of nuclear Ki67, a well known indicator of cellular proliferation (unpublished results). Relating back to tissue homeostasis, these observations may indicate that increased cellular turnover, as opposed to either manifestations of apoptosis or cellular proliferation taken alone, may be of relevance to the aggressiveness of PCa and potentially other cancers. However, as we cannot directly correlate NOXA or PUMA expression with apoptosis per se, it will be interesting to address this possibility more directly. For example, this could be done by co-assessing activated caspase-3 expression in parallel with Ki67 in the same tissues using immunofluorescence. The ratio of these two markers in combination could then be confronted to the onset of BCR.

The survival tree analyses used in this study allowed us to sketch the relationship between NOXA, PUMA, clinico-pathological parameters and BCR. Using this method, we generated models and ranked them according to their predictive accuracy as determined by the IBS. In terms of prognosis, the top-ranking models were particularly apt at identifying patients at very high or very low risk of BCR. For example, in the top-ranking model, no patients relapsed following surgery within the follow up period (0/13) whereas 14/15 patients relapsed in the worst prognostic group (Chapter III, Fig. 5a). In other words, this model allows us to say with very high certainty (over 95%) whether 28 of the 62 patients present in the cohort will relapse or not. Hence, if this model were to be used in a clinical setting, it would be useful for patients in the extreme prognostic groups at very low or very high risk of relapse (45% of patients in this case), while not very informative for the rest (55%) of patients in the intermediate risk groups. As such, it may be argued that the best prognostic model in a clinical setting is not the one that can most accurately stratify patient risk but rather the one that can predict the most patients at very high and very low risk. Although survival trees as well as other modeling methods including multivariate Cox models and nomograms provide a step in the right direction, they do not account for this subtlety. However, until the emergence of such an ideal marker or

prognostic model, it remains important to further evaluate currently promising models as these are more likely to aid patients in the near future.

6.3. The implications of androgen receptor expression and sub-cellular localization in the development of prostate cancer.

As the observations made in chapter II suggested that AR expression could have an impact on the efficacy of IP6 we set out to explore the expression and sub-cellular localization of the AR in prostate tissues. In light of conflicting results presented in the literature regarding the link between AR expression and PCa progression prior to treatment by androgen deprivation, we also aimed to assess the association between AR expression and BCR following radical prostatectomy. In chapter IV, we used a similar strategy as in chapter III to assess the expression of both cytoplasmic and nuclear AR in prostate tissues, and to explore the relationship between AR expression, clinico-pathological features and BCR. We found that the most striking change in nuclear AR (as well as nuclear/cytoplasmic AR ratio) was observed when comparing normal prostate tissues obtained from cancer-free patients to non-neoplasic tissues obtained from PCa patients having undergone radical prostatectomy. In contrast, cytoplasmic AR staining intensity was particularly elevated in AIPCa specimens. We also found that assessed alone, low cytoplasmic AR staining intensities, low nuclear AR staining intensities, and high nuclear/cytoplasmic AR staining intensity ratios were all associated to BCR. In a multivariate Cox model including Gleason score, preoperative PSA, surgical margin status and pathological stage, we found that low cytoplasmic AR remained an independent predictor of BCR. However, similar multivariate analyses using survival trees showed a complex relationship between AR expression, clinico-pathological features and BCR.

The results presented in Chapter IV bring forth the notion that increased nuclear activity of the AR may be an early event in the development of PCa

(Chapter IV, Fig. 2 a,c). However, it remains unclear whether increased nuclear expression of the AR is a cause or a consequence of the progression of PCa in its early stages. To answer this question, transgenic mouse models will likely prove to be useful tools. As mentioned in section 1.3.4.3 and as discussed in chapter IV, transgenic mice that have lost the expression of NKX3.1 exhibit increased AR expression [343]. Hence, it would be interesting to see whether knockdown of the AR, for example using a shRNA under the control of an inducible promoter, could reduce the epithelial dysplasia and hyperplasia observed normally in the development of NKX3.1 knockout mice [342]. A more ambitious project would be to generate transgenic mice harboring androgen-responsive oncogenic TMRPSS2-ETS fusions. These mice could then be crossed with other transgenic models such as mice over-expressing the AR specifically in the prostate [345] or expressing an inducible shRNA targeted against the AR as described above. Such models may shed some light on the implication of the AR in what are potentially the early stages of prostate carcinogenesis [604].

Although the results presented in chapter IV highlight the potential involvement of nuclear AR during the initial stages of PCa, they provide a mixed view of whether the AR could be useful as a prognostic marker, particularly when confronted with relevant clinico-pathological parameters. Although multivariate Cox analyses suggest that cytoplasmic AR in particular is an independent predictor of BCR, survival tree analyses paint a more complex picture. Whereas recurrent tree structures retaining surgical margin status and NOXA as prognostic classifiers were overwhelmingly present in the top-ranking models described in chapter III, no such trend was observed for the AR in chapter IV. Instead, we observed that the AR predicted different outcomes, particularly in relation to the surgical margin status and clinico-pathological stage of the tumors. Altogether, these data suggest that the AR may hold only limited additional prognostic information.

One important issue raised in chapter IV is the potential influence of artifacts on the interpretation of results obtained using immunohistochemistry.

Indeed, there is a pressing need to develop ways to ensure that observed differences in tissue staining intensities (as assessed by immunohistochemtry) are truly due to differences in protein expression and not linked to differences in tissue collection and fixation methods or to the age of the archival specimens. To resolve this problem at the source will undoubtedly require a considerable coordination effort between clinicians, pathologists, and researchers so as to establish a standardized protocol for collection and fixation of samples to be used. This problem applies both to prospective immunochemistry studies (such as those done in chapters III and IV) as well as to potential future diagnostic and prognostic applications.

Normalization using an internal control could be a valid alternative solution to this problem. In chapter IV, we used the ratio of nuclear to cytoplasmic AR staining as an additional parameter and found markedly different results as compared to nuclear or cytoplasmic AR expression taken alone. Of course, as nuclear and cytoplasmic AR are not independent of one another, the nuclear/cytoplasmic AR staining intensity ratio cannot be truly considered as an internal control. Hence, developing novel methods of internal normalization in immunohistochemistry is likely a worthwhile pursuit. However, like for any normalization procedure, one must be fairly certain that the internal reference does not fluctuate in parallel with the gene/protein of interest. This poses a particular challenge in immunohistochemistry as even though the expression of a given protein may be similar across different cells, its sub-cellular distribution (and consequently visual densitometric properties) may change. Nonetheless, some indications suggest that our observations are not merely artifacts. For example, we can see that AIPCa tissues from patients that have undergone androgen deprivation therapy exhibit much higher levels of cytoplasmic AR than all other tissues not subjected to this treatment. This finding is in agreement with the effect of androgen deprivation on the sub-cellular localization of the AR observed using other methods [605]. In addition, other studies have observed increased overall AR expression in AIPCa tissues as we have reported in chapter

IV [363, 436, 606]. In relation to both chapter III and IV, the fact that NA tissues, PIN, and cancerous PCa tissues were taken from the same RP specimens strongly argue against the possibility that the observed differences in tissue staining between these tissue types is linked to variations in tissue fixation methods or to the age of the specimen. On the other hand, we cannot exclude the possibility that specimens obtained form autopsied cancer-free patients may exhibit differences solely based on differences in patient age or *post mortum* state of the patient. However, in the case of the AR, the nuclear/cytoplasmic staining intensity ratio should have theoretically been similar between the cancer-free tissues and NA tissues if it were not for actual biological differences (Chapter IV Fig 2c).

In chapter IV, we found that essentially all prostate tissues expressed the AR although overall expression appeared to increase with the progression towards AIPCa. Considering that several AIPCa cell lines, including PC3 and DU145 cells, do not express the AR, this finding is somewhat surprising. At the same time, it must be noted that both PC3 and DU145 cells were obtained from metastases to the bone and brain respectively (see sections 1.3.6.1 and 1.3.6.2). In contrast, all of the AIPCa specimens used in chapters III and IV were obtained from trans-urethral resections of the prostate (TURP). Hence, we cannot exclude the possibility that distant metastatic AIPCa tumors are more frequently negative for the AR. Supporting this may indeed be the case, one recent study has found that although strong expression of the AR was evident in 100% of localized PCa, this number deceased to 80% in lymph node metastases and to 40% in distant hormone-refractory metastases [607]. Although access to metastatic AIPCa samples is difficult, it will be interesting to confirm these findings by studying nuclear and cytoplasmic AR expression in function of the site of metastasis.

In chapter II, we suggested that IP6 sensitivity may be linked to AR expression. Hence, in the eventuality where IP6 could be used for treatment of human PCa, we would expect from this suggestion that IP6 would likely have the most effect when AR expression is at its lowest. Although a significant proportion

of distant metastases may also exhibit lowered AR expression, the results presented in chapter IV suggest that AR expression is at its lowest during the initial stages of PCa progression. Hence, as mono-therapy, this argues in favor of using IP6 for chemoprevetion of PCa. To test this hypothesis, we have recently initiated a study using the transgenic adenocarcinoma of the mouse prostate (TRAMP) transgenic mouse model. TRAMP mice have been engineered to express oncogenic SV40 antigens specifically in the prostate and are the best characterized and most widely used model for PCa chemoprevention studies. Heterozygous TRAMP mice develop localized tumors of both basal/luminal and neuroendocrine origin beginning at approximately 8-10 weeks of age and by 28 weeks, 100% of mice exhibit signs of distant metastasis, mostly in the lymph nodes and lungs. TRAMP metastases notably express little to no AR and are generally of neuroendocrine origin [608-611]. These mice will be provided with 2% IP6 in the drinking water and tumor progression will be compared to that of control mice. Using this model, we also aim to compare the effect of androgen-depletion by surgical castration with and without IP6 provided in the drinking water. The underlying rationale to this aim is that whereas androgen-deprivation necessarily targets AR-positive cells, the action of IP6 could potentially complement androgen-deprivation by targeting AR-negative cells.

As mentioned above, although TRAMP mice develop localized tumors of both basal/luminal and neuroendocrine origin, TRAMP metastases express little to no AR and are generally of neuroendocrine origin. In the human disease, neuroendocrine tumors are thought to be less frequent. Hence, it will be important to eventually use other transgenic mouse models if promising results are obtained using IP6 as a chemopreventive agent in the TRAMP model. To this effect, the prostate-specific conditional PTEN knockout model could be used. These mice exhibit a prostate-specific deletion in the phosphatase domain of the PTEN tumor suppressor. As mentioned in section 1.2.4.1.2, PTEN is a negative regulator of Akt, which phosphorylates key proteins involved in cell proliferation and survival such as IKKs, BAD and mTOR. Homozygous PTEN conditional knockout mice begin to develop invasive adenocarcinoma by 9-weeks of age and, similarly to

TRAMP mice, begin to exhibit lymph node and pulmonary metastases at approximately 12 weeks. In contrast to TRAMP mice, metastases from PTEN conditional knockout mice express the AR and do not originate from neuroendocrine cells [335]. It may also be interesting to use NKX3.1 knockout and NKX3.1 / PTEN double knockout mice as well, since the loss of both NKX3.1 and PTEN are thought to be early events in the progression of localized PCa [604].

6.4. IP6 in combination with proteasome inhibitors: mechanistic and therapeutic implications

While the data presented in chapter II warranted further studying the role of the AR in modulating the efficacy of IP6, it was clear from the multitude of AR-interacting proteins presented in section 1.3.5 that advancing our knowledge of the mechanisms of IP6 in PC3 cells would be necessary prior to this endeavor. Hence, with the underlying goal of innovating in the treatment of PCa, we continued our exploration of the molecular mechanisms of IP6 in PC3 cells based on the evidence obtained in chapter II. We found that although classical NF-κB subunits translocated to the nucleus in response to IP6, thwarting NF-κB-mediated transcription had little effect on the efficacy of IP6. However, we found that treating cells with IP6 caused temporal fluctuations in the expression and ratios of pro and anti-apoptotic BCL-2 family proteins, wherein levels of pro-apoptotic PUMA, BIK/NBK and NOXA rose early-on (between 4-8 hours post treatment) and levels of anti-apoptotic MCL-1 and BCL-2 decreased after 24 hours. Suggesting that the cytotoxic effects of IP6 may not depend on transcriptional events, we found that PC3 cells treated with a transcription inhibitor (actinomycin D) did not exhibit significant changes in PC3 cell sensitivity to IP6. However, suggesting the potential involvement of pro-apoptotic BCL-2 family proteins, we found that inhibiting protein translation by pre-treating PC3 cells with cycloheximide had a significant protective effect. In light of this, we reasoned that blocking proteasome-mediated protein degradation might

sensitize cells to the effects of IP6 by further increasing the pro to anti-apoptotic BCL-2 family protein ratio. We found that the proteasome inihibitor MG-132 sensitized both AR-negative PC3 and DU145 AIPCa cell lines to the effects of IP6. The enhanced effect of combining IP6 with proteasome inhibitors was found to implicate mitochondrial outer membrane permeation (MOMP) and required protein translation. Suggesting a role of pro-apoptotic BCL-2 family members in this phenomenon, MG-132 led to increased protein levels of several pro-apoptotic BCL-2 family members. Albeit slightly, the levels of these proteins was further increased by addition of IP6 whereas co-treatment with cycloheximide completely blocked this effect, with the notable exception of PUMA.

The results obtained in chapter V suggest the involvement of BCL-2 family members in the response to IP6 and in the response to the combination of IP6 with MG-132. However, it is unlikely that the observed combined effect of IP6 and proteasome inhibitors can be explained solely on the observed modulations in NOXA, BIK/NBK, and PUMA pro-apoptotic protein levels. As introduced in sections 1.2.3.1.1 trough 1.2.3.2.3, there are over 20 known pro-apoptotic and at least 10 known anti-apoptotic members of the BCL-2 family proteins. In addition, many of these proteins can be further modified by post-translational modification. Hence, it will be necessary to probe for modulations in other BCL-2 family members, as well as for potential post-translational modifications to get a clear picture of which proteins may be particularly relevant to the results observed in chapter V.

Given the large number of known and potentially unknown BCL-2 family members, probing for each of them individually by Western blotting is likely not the best strategy, particularly as potentially novel candidates cannot be found using this method. As such, a proteomics approach may be more promising. For example, PC3 cells could be treated with IP6, MG-132, MG-132 and IP6, as well as with a vehicle control. Whole-cell protein extracts could then be obtained and subjected to HPLC-based peptide separation methods such as MudPIT (multi-

dimensional protein identification technology) combined with sample tagging methods such as I-TRAQ (isobaric tags for relative and absolute quantification) that also facilitate the identification of posttranslational modifications and the comparison of up to four samples simultaneously [612, 613]. By quantifying and comparing the peptide signatures of IP6/MG-132 co-treated cells to cells treated with either IP6 or MG-132 obtained by mass spectrometry, differentially expressed peptides (or post-translationally modified peptides) could then be identified. Since there is clear evidence that the enhanced effect observed when combining IP6 with MG-132 involves mitochondrial depolarization, it would be also interesting to do similar experiments using mitochondrial extracts instead of whole cell extracts. As such, in addition to providing more direct information on what proteins may be involved in the observed effects on MOMP, the potentially large number of candidates that can be expected to be obtained using a proteomics approach may be reduced.

One possibility brought forth in chapter V is that IP6 may function by a transcription-independent mechanism. Although the observed down-regulation of MCL-1 and BCL-2 after 24 hours of treatment with IP6 argues against this idea, one possibility is that IP6 stabilizes the expression of pro-apoptotic BCL-2 family members by inhibiting proteasome activity, at least during the first 4 to 8 hours of treatment. This hypothesis may be tested directly by measuring proteasome activity in whole cell extracts after treatment of PC3 cells with IP6. To this effect, fluorigenic substrates measuring chymotrypsin-like activity (Succ-LLVY-AMC), trypsin-like activity (Z-ARR-AMC), and peptidylglutamyl peptide hydrolyzing activity (Z-LLE-AMC) can be used [614]. Using these substrates, it would also be interesting to measure proteasome activity upon treatment of PC3 cells with MG-132 and with a combination of IP6 and MG-132 to assess whether co-treatment leads to further inhibition of proteasome activity as this could help to explain some of the results observed in chapter V.

Along the same line of thought as above, IP6 could potentially stabilize pro-apoptotic proteins by inhibiting their poly-ubiquitination and targeting for proteasome-mediated degradation. With respect to this idea, it is known that the anti-apoptotic protein MCL-1 is targeted for degradation by the E3 ubiquitin conjugating protein MULE/ARF-BP1 [38]. Interestingly, looking at the response of MCL-1, NOXA, and BIK/NBK levels to treatment of PC3 cells with MG-132 and cycloheximide reveals a similar pattern, suggesting that analogously to MCL-1, NOXA and BIK/NBK may be actively targeted for degradation by ubiquitin ligases (Chapter V, Fig 6). Beyond MULE/ARFBP1, it is quite possible that other such proteins exist that instead target NOXA and BIK/NBK for degradation by the proteasome. The activity of these hypothetical proteins could potentially be inhibited by IP6. Interestingly, such a mechanism may also explain why others have observed increased $p21^{CIP1}$ and $p27^{KIP1}$ expression as well as cell cycle arrest in DU145 cells treated by IP6 [585]. As mentioned in section 1.2.2.3 the degradation of both $p21^{CIP1}$ and $p27^{KIP1}$ is regulated by SCF ubiquitin ligases.

Another possibility discussed in chapter V is that IP6 leads to increased protein translation by modulating the activity of pathways that control protein translation. Such pathways include the well known Akt/mTOR pathway or the recently identified RalA pathway. RalA is an effector of Ras signaling that is involved in membrane trafficking, actin organization, and gene expression [615-617]. Activation of RalA was found to lead to decreased expression of the anti-apoptotic protein $FLIP_S$, which inhibits extrinsic apoptosis (such as that induced by TRAIL) by associating to the DISC (see section 1.2.3.3.1) [618]. The effect of RalA on $FLIP_S$ levels was found to be mediated by the inhibition of cdc42 that normally activates S6K1, a kinase that is involved in the control of ribosomal translation. Cdc42 and S6K1 are also downstream targets of mTOR signaling (see section 1.2.2.2). Hence, it will be interesting to determine whether modulation mTOR, RalA or of Cdc42 and S6K1 that are common to both mTOR or RalA pathways can modulate the observed changes in PUMA, BIK/NBK and NOXA levels induced by treatment with IP6 and whether the efficacy of IP6 can be

modulated in consequence. This could be done, for example, by using siRNAs targeting mTOR, RalA, S6K1 or cdc42. Additionally, it may be interesting to evaluate the combined effect of mTOR inhibitors such as CCI-779 [518] with IP6, particularly considering that drugs such as CCI-779 are already undergoing clinical evaluation for renal cancer and are currently at the pre-clinical stage for treatment of prostate cancer.

In light of the evidence presented in the literature suggesting that IP6 may play a role in mRNA export through the TAP/NFX1 pathway (see sections 1.1.3 and 1.4.4.1.3), a most intriguing possibility is that IP6 increases the export of accumulated nuclear transcripts towards the cytoplasm. Although we would expect that such a mechanism should also lead to increased expression of various transcripts and not just pro-apoptotic BCL-2 family members, two observations prevent the rejection of this hypothesis. First, in absence of actinomycin D, we observe increased mRNA expression of *PUMA*, *NOXA* and *BAX*, detectable as early as 4 hours post-treatment (Chapter II, Fig 5, and unpublished data). Hence, if mRNA export were to be increased in these conditions, we would expect that more of these pro-apoptotic mRNAs would be translated once in the cytoplasmic compartment, which could effectively shift the balance of pro-apoptotic to anti-apoptotic proteins. Second, a 24-hour treatment of PC3 cells with actinomycin D reduces the basal mRNA expression levels of BCL-2 and BCL-XL to less than 5% the levels of the control, whereas the basal levels of NOXA, PUMA and BAX remain significantly higher (65%, 15% and 14% respectively, unpublished data). Therefore, increased mRNA export induced by IP6 could still lead to a shift in the ratio of pro-apoptotic to anti-apoptotic BCL-2 family proteins in this context. As such, one possible way to test whether IP6 increases mRNA export is to use realtime PCR in combination with subcellular fractionation. Specifically, PC3 cells could be treated with IP6 or with control and both cytoplasmic and nuclear fractions could be prepared. RNA could then be extracted from both nuclear and cytoplasmic fractions and subsequently converted to cDNA. The relative nuclear/cytoplasmic expression of pro-apoptotic genes could then be evaluated

using realtime PCR, similarly to what was done in chapters II and V. Notably, such a procedure has been used successfully by others to demonstrate the ability of VSV proteins to block nuclear mRNA export in infected cells [619].

Another intriguing point of discussion brought forth in chapter V is the possibility that certain BCL-2 family proteins such as PUMA may be translated in part by mitochondrial ribosomes. This idea stems mainly from the observation that cycloheximide does not appear to completely prevent observed IP6-induced increases in protein levels of PUMA (Chapter V, Fig. 6). As discussed in chapter V, cycloheximide is known to be ineffective against mitochondrial ribosomes [620], which are of different composition than their cytoplasmic counterparts (see sections 1.1.3 and 1.2.3.1). However, other translation inhibitors such as chloramphenicol are selective for mitochondrial ribosomes [620]. Hence it will be interesting to compare the effects of cycloheximide and chloramphenicol on the effects of IP6, both at the level of cytotoxicity and expression of BCL-2 family members.

Analogously to what was observed in chapter II we found that the proteasome inhibitor MG-132 sensitized AR-negative PC3 and DU145 AIPCa cells to the effects of IP6 but in contrast had little effect on the sensitivity of AR-positive, androgen-responsive LNCaP and 22Rv1 cells to IP6 (Chapter V, Fig 4a-e). This further highlights what may be potentially deep-rooted differences between AR-negative and AR-positive cells. It is tempting to speculate that this may have some link to the expression profiles of BCL-2 family members in AR-negative and AR-positive cells, as hinted from the results obtained for NOXA and PUMA in chapter III (Chapter III, Fig.1). A first step could be to assess the effect of down-regulating NOXA or PUMA expression on the sensitivity to IP6 and combined IP6/MG-132 treatment. However, since it is likely that down-regulation of a single protein may not be sufficient to elicit an effect because of the highly redundant nature of BCL-2 family proteins, it will also be interesting to characterize the expression of BCL-2 family members in DU145, LNCaP and

22Rv1 cells as was done in chapter V for PC3 cells. As the differences observed between these cell lines could potentially be due to their different cellular origins, it would be equally interesting to determine whether there is any difference *in vivo* between basal cells, neuroendocrine cells, and epithelial cells, with respect to BCL-2 family member expression. This could be achieved using laser capture microscopy, where single basal, neuroendocrine, and epithelial cells could be isolated from frozen radical prostatectomy or even autopsy specimens, following immunohistochemical staining for differentiation markers such as those described in section. RNA could be subsequently extracted from these three cellular sub-types and screened for the expression of BCL-2 family members either by RNA microarray and/or by realtime PCR.

Following from our suggestion that pre-clinical evaluation of combined treatment with IP6 and proteasome inhibitors may be warranted, we have recently initiated an *in vivo* study using PC3 xenografts implanted subcutaneously in immunocompromised mice. In the context of the human disease, this model is most analogous to recurrent cancer, as tumors arise from a relatively small number of "remaining" cells. Using this model, we aim to assess whether IP6 can delay the progression of PC3 tumors when provided in the drinking water (2% w/v as done in [558] for DU145 cells). In addition, to test the combined effect of IP6 and MG-132, PC3 tumors will be allowed to grow to a pre-determined size and will then be challenged with MG-132. Of the mice treated with MG-132, a subset will be provided with water containing a 2% solution of IP6. If these trials are successful, it will be interesting to see whether a similar effect can be obtained using DU145 cells and using other models, such as intra-cardiac injection, that lead to widespread metastasis [621]. Eventually, it will also be relevant to test whether the FDA and EMEA approved Bortezomib can be used instead of MG-132 or ALLN, which we believe to be a likely possibility. In the event that these pre-clinical trials are successful, it seems feasible that clinical trials could be initiated fairly rapidly as Bortezomib is already undergoing clinical trials for treatment of AIPCa in combination with Docetaxel.

6.5. Conclusion

The over-arching goal of this doctoral project was to further our understanding of the factors involved in both therapeutic response and clinical outcome, and to innovate in the treatment of PCa. Our studies on IP6 have provided a backbone to this effect. With respect to understanding the factors involved in therapeutic response, we are among the first to have brought to light the concept that androgen-independent functions of the AR may be involved in conferring resistance to AIPCa against certain types of therapy, exemplified by the decreased sensitivity of AR-positive cells to the effects of IP6. In relation to our understanding of the factors involved in clinical outcome of prostate cancer, we have contributed both through the application of emerging methods in multi-marker modeling and by discovering the prognostic potential of NOXA. In addition, we have brought forth evidence to suggest that nuclear AR activities may be involved in the early stages of PCa and have highlighted the relevance of studying cytoplasmic functions of the AR in PCa and AIPCa *in vivo*. Finally, in relation to innovation in the treatment of PCa, our studies on the mechanisms of IP6 have led to the discovery that combined treatment with IP6 and proteasome inhibitors can enhance cytotoxicity against AIPCa cells. Collectively with the future studies we hope to have inspired, we conclude that the innovative concepts and methods, the novel prognostic markers and models, as well as the new treatment strategies proposed during the course of this doctoral project could eventually lead to the better management of PCa.

REFERENCES

1. institute, W.R. *Earthtrends: Environmental Information.* 2007 [cited; Available from: http://earthtrends.wri.org.
2. Klevecz, R.R., *Temporal coordination of DNA replication with enzyme synthesis in diploid and heteroploid cells.* Science, 1969. **166**(3912): p. 1536-8.
3. Langston, L.D. and M. O'Donnell, *DNA replication: keep moving and don't mind the gap.* Mol Cell, 2006. **23**(2): p. 155-60.
4. Dianov, G.L. and J.L. Parsons, *Co-ordination of DNA single strand break repair.* DNA Repair (Amst), 2007. **6**(4): p. 454-60.
5. Counis, M.F. and A. Torriglia, *DNases and apoptosis.* Biochem Cell Biol, 2000. **78**(4): p. 405-14.
6. Fisher, A.E., et al., *Poly(ADP-ribose) polymerase 1 accelerates single-strand break repair in concert with poly(ADP-ribose) glycohydrolase.* Mol Cell Biol, 2007. **27**(15): p. 5597-605.
7. Vaish, M., *Mismatch repair deficiencies transforming stem cells into cancer stem cells and therapeutic implications.* Mol Cancer, 2007. **6**: p. 26.
8. Iliakis, G., et al., *Mechanisms of DNA double strand break repair and chromosome aberration formation.* Cytogenet Genome Res, 2004. **104**(1-4): p. 14-20.
9. Blasco, M.A., *The epigenetic regulation of mammalian telomeres.* Nat Rev Genet, 2007. **8**(4): p. 299-309.
10. Efeyan, A. and M. Serrano, *p53: guardian of the genome and policeman of the oncogenes.* Cell Cycle, 2007. **6**(9): p. 1006-10.
11. Campbell, N., *Biology.* 1993: Benajamin/Cummings Publishing Company Inc.
12. Russell, J. and J.C. Zomerdijk, *The RNA polymerase I transcription machinery.* Biochem Soc Symp, 2006(73): p. 203-16.
13. Phatnani, H.P. and A.L. Greenleaf, *Phosphorylation and functions of the RNA polymerase II CTD.* Genes Dev, 2006. **20**(21): p. 2922-36.

14. Haeusler, R.A. and D.R. Engelke, *Spatial organization of transcription by RNA polymerase III.* Nucleic Acids Res, 2006. **34**(17): p. 4826-36.

15. Dillon, N., *Heterochromatin structure and function.* Biol Cell, 2004. **96**(8): p. 631-7.

16. Li, B., M. Carey, and J.L. Workman, *The role of chromatin during transcription.* Cell, 2007. **128**(4): p. 707-19.

17. Villar-Garea, A. and A. Imhof, *The analysis of histone modifications.* Biochim Biophys Acta, 2006. **1764**(12): p. 1932-9.

18. Gallinari, P., et al., *HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics.* Cell Res, 2007. **17**(3): p. 195-211.

19. Gregory, R.I., et al., *Human RISC couples microRNA biogenesis and posttranscriptional gene silencing.* Cell, 2005. **123**(4): p. 631-40.

20. Fuchs, U., C. Damm-Welk, and A. Borkhardt, *Silencing of disease-related genes by small interfering RNAs.* Curr Mol Med, 2004. **4**(5): p. 507-17.

21. Fernandez-Silva, P., J.A. Enriquez, and J. Montoya, *Replication and transcription of mammalian mitochondrial DNA.* Exp Physiol, 2003. **88**(1): p. 41-56.

22. Gerbi, S.A., A.V. Borovjagin, and T.S. Lange, *The nucleolus: a site of ribonucleoprotein maturation.* Curr Opin Cell Biol, 2003. **15**(3): p. 318-25.

23. Rodriguez, M.S., C. Dargemont, and F. Stutz, *Nuclear export of RNA.* Biol Cell, 2004. **96**(8): p. 639-55.

24. Proud, C.G., *Signalling to translation: how signal transduction pathways control the protein synthetic machinery.* Biochem J, 2007. **403**(2): p. 217-34.

25. Schmelzle, T. and M.N. Hall, *TOR, a central controller of cell growth.* Cell, 2000. **103**(2): p. 253-62.

26. Gronostajski, R.M., A.B. Pardee, and A.L. Goldberg, *The ATP dependence of the degradation of short- and long-lived proteins in growing fibroblasts.* J Biol Chem, 1985. **260**(6): p. 3344-9.

27. Lee, D.H. and A.L. Goldberg, *Proteasome inhibitors: valuable new tools for cell biologists.* Trends Cell Biol, 1998. **8**(10): p. 397-403.

28. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.

29. Zhang, H.G., et al., *Regulation of apoptosis proteins in cancer cells by ubiquitin.* Oncogene, 2004. **23**(11): p. 2009-15.

30. Marchenko, N.D., et al., *Monoubiquitylation promotes mitochondrial p53 translocation.* Embo J, 2007. **26**(4): p. 923-34.

31. Sarcinella, E., et al., *Mono-ubiquitylation of H2A.Z distinguishes its association with euchromatin or facultative heterochromatin.* Mol Cell Biol, 2007.

32. Vodermaier, H.C., *APC/C and SCF: controlling each other and the cell cycle.* Curr Biol, 2004. **14**(18): p. R787-96.

33. Honda, R., H. Tanaka, and H. Yasuda, *Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53.* FEBS Lett, 1997. **420**(1): p. 25-7.

34. Karin, M. and Y. Ben-Neriah, *Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity.* Annu Rev Immunol, 2000. **18**: p. 621-63.

35. Yu, Z.K., J.L. Gervais, and H. Zhang, *Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins.* Proc Natl Acad Sci U S A, 1998. **95**(19): p. 11324-9.

36. Tsvetkov, L.M., et al., *p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27.* Curr Biol, 1999. **9**(12): p. 661-4.

37. Binne, U.K., et al., *Retinoblastoma protein and anaphase-promoting complex physically interact and functionally cooperate during cell-cycle exit.* Nat Cell Biol, 2007. **9**(2): p. 225-32.

38. Zhong, Q., et al., *Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis.* Cell, 2005. **121**(7): p. 1085-95.

39. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. **432**(7015): p. 316-23.

40. Maddika, S., et al., *Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy.* Drug Resist Updat, 2007. **10**(1-2): p. 13-29.

41. Vermeulen, K., D.R. Van Bockstaele, and Z.N. Berneman, *The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer.* Cell Prolif, 2003. **36**(3): p. 131-49.

42. Assoian, R.K. and X. Zhu, *Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression.* Curr Opin Cell Biol, 1997. **9**(1): p. 93-8.

43. Vidal, A. and A. Koff, *Cell-cycle inhibitors: three families united by a common cause.* Gene, 2000. **247**(1-2): p. 1-15.

44. DeGregori, J., *The genetics of the E2F family of transcription factors: shared functions and unique roles.* Biochim Biophys Acta, 2002. **1602**(2): p. 131-50.

45. Stevaux, O. and N.J. Dyson, *A revised picture of the E2F transcriptional network and RB function.* Curr Opin Cell Biol, 2002. **14**(6): p. 684-91.

46. Dimova, D.K. and N.J. Dyson, *The E2F transcriptional network: old acquaintances with new faces.* Oncogene, 2005. **24**(17): p. 2810-26.

47. Dyson, N., *The regulation of E2F by pRB-family proteins.* Genes Dev, 1998. **12**(15): p. 2245-62.

48. Nevins, J.R., *Toward an understanding of the functional complexity of the E2F and retinoblastoma families.* Cell Growth Differ, 1998. **9**(8): p. 585-93.

49. Karin, M., et al., *NF-kappaB in cancer: from innocent bystander to major culprit.* Nat Rev Cancer, 2002. **2**(4): p. 301-10.

50. Rowlands, T.M., et al., *Beta-catenin and cyclin D1: connecting development to breast cancer.* Cell Cycle, 2004. **3**(2): p. 145-8.

51. Ohtani, K., *Implication of transcription factor E2F in regulation of DNA replication.* Front Biosci, 1999. **4**: p. D793-804.

52. Hannon, G.J. and D. Beach, *p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest.* Nature, 1994. **371**(6494): p. 257-61.

53. el-Deiry, W.S., et al., *WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis.* Cancer Res, 1994. **54**(5): p. 1169-74.

54. Kay, J.E., et al., *Inhibition of T and B lymphocyte proliferation by rapamycin.* Immunology, 1991. **72**(4): p. 544-9.

55. Nakayama, K.I. and K. Nakayama, *Regulation of the cell cycle by SCF-type ubiquitin ligases.* Semin Cell Dev Biol, 2005. **16**(3): p. 323-33.

56. Lin, D.I., et al., *Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex.* Mol Cell, 2006. **24**(3): p. 355-66.

57. van Drogen, F., et al., *Ubiquitylation of cyclin E requires the sequential function of SCF complexes containing distinct hCdc4 isoforms.* Mol Cell, 2006. **23**(1): p. 37-48.

58. Castro, A., et al., *The anaphase-promoting complex: a key factor in the regulation of cell cycle.* Oncogene, 2005. **24**(3): p. 314-25.

59. Coutts, A.S. and N.B. La Thangue, *Mdm2 widens its repertoire.* Cell Cycle, 2007. **6**(7): p. 827-9.

60. Schwartzman, R.A. and J.A. Cidlowski, *Apoptosis: the biochemistry and molecular biology of programmed cell death.* Endocr Rev, 1993. **14**(2): p. 133-51.

61. Wyllie, A.H., *Apoptosis: an overview.* Br Med Bull, 1997. **53**(3): p. 451-65.

62. Fulda, S. and K.M. Debatin, *Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy.* Oncogene, 2006. **25**(34): p. 4798-811.

63. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.

64. Ravagnan, L., T. Roumier, and G. Kroemer, *Mitochondria, the killer organelles and their weapons.* J Cell Physiol, 2002. **192**(2): p. 131-7.

65. Cunha, G.R., et al., *Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development.* J Steroid Biochem Mol Biol, 2004. **92**(4): p. 221-36.

66. Fromigue, O., D. Modrowski, and P.J. Marie, *Apoptosis in membranous bone formation: role of fibroblast growth factor and bone morphogenetic protein signaling.* Crit Rev Eukaryot Gene Expr, 2005. **15**(1): p. 75-92.

67. Logan, D.C., *The mitochondrial compartment.* J Exp Bot, 2006. **57**(6): p. 1225-43.

68. Robert, D., *Elements de biologie cellulaire.* 1998: Doin.

69. Taanman, J.W., *The mitochondrial genome: structure, transcription, translation and replication.* Biochim Biophys Acta, 1999. **1410**(2): p. 103-23.

70. Verner, K., *Co-translational protein import into mitochondria: an alternative view.* Trends Biochem Sci, 1993. **18**(10): p. 366-71.

71. Kamenski, P., et al., *Evidence for an adaptation mechanism of mitochondrial translation via tRNA import from the cytosol.* Mol Cell, 2007. **26**(5): p. 625-37.

72. Bellot, G., et al., *TOM22, a core component of the mitochondria outer membrane protein translocation pore, is a mitochondrial receptor for the proapoptotic protein Bax.* Cell Death Differ, 2007. **14**(4): p. 785-94.

73. Szabo, I., V. De Pinto, and M. Zoratti, *The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel.* FEBS Lett, 1993. **330**(2): p. 206-10.

74. Szabo, I. and M. Zoratti, *The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore.* FEBS Lett, 1993. **330**(2): p. 201-5.

75. Hunter, D.R., R.A. Haworth, and J.H. Southard, *Relationship between configuration, function, and permeability in calcium-treated mitochondria.* J Biol Chem, 1976. **251**(16): p. 5069-77.

76. Vayssiere, J.L., et al., *Commitment to apoptosis is associated with changes in mitochondrial biogenesis and activity in cell lines conditionally immortalized with simian virus 40.* Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11752-6.

77. Zoratti, M. and I. Szabo, *The mitochondrial permeability transition.* Biochim Biophys Acta, 1995. **1241**(2): p. 139-76.

78. Kroemer, G. and J.C. Reed, *Mitochondrial control of cell death.* Nat Med, 2000. **6**(5): p. 513-9.

79. Crompton, M., *The mitochondrial permeability transition pore and its role in cell death.* Biochem J, 1999. **341 (Pt 2)**: p. 233-49.

80. Halestrap, A.P., G.P. McStay, and S.J. Clarke, *The permeability transition pore complex: another view.* Biochimie, 2002. **84**(2-3): p. 153-66.

81. Kluck, R.M., et al., *The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis.* Science, 1997. **275**(5303): p. 1132-6.

82. Yang, J., et al., *Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked.* Science, 1997. **275**(5303): p. 1129-32.

83. Susin, S.A., et al., *Molecular characterization of mitochondrial apoptosis-inducing factor.* Nature, 1999. **397**(6718): p. 441-6.

84. Suzuki, Y., et al., *A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death.* Mol Cell, 2001. **8**(3): p. 613-21.

85. Du, C., et al., *Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition.* Cell, 2000. **102**(1): p. 33-42.

86. Verhagen, A.M., et al., *Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins.* Cell, 2000. **102**(1): p. 43-53.

87. Li, L.Y., X. Luo, and X. Wang, *Endonuclease G is an apoptotic DNase when released from mitochondria.* Nature, 2001. **412**(6842): p. 95-9.

88. Parrish, J., et al., *Mitochondrial endonuclease G is important for apoptosis in C. elegans.* Nature, 2001. **412**(6842): p. 90-4.

89. Krajewski, S., et al., *Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5752-7.

90. Mancini, M., et al., *The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling.* J Cell Biol, 1998. **140**(6): p. 1485-95.

91. Qin, Z.H., et al., *Pro-caspase-8 is predominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation.* J Biol Chem, 2001. **276**(11): p. 8079-86.

92. Susin, S.A., et al., *Mitochondrial release of caspase-2 and -9 during the apoptotic process.* J Exp Med, 1999. **189**(2): p. 381-94.

93. Adams, J.M. and S. Cory, *Life-or-death decisions by the Bcl-2 protein family.* Trends Biochem Sci, 2001. **26**(1): p. 61-6.

94. Cleary, M.L., S.D. Smith, and J. Sklar, *Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation.* Cell, 1986. **47**(1): p. 19-28.

95. Boise, L.H., et al., *bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death.* Cell, 1993. **74**(4): p. 597-608.

96. Schmitt, E., et al., *Bcl-xES, a BH4- and BH2-containing antiapoptotic protein, delays Bax oligomer formation and binds Apaf-1, blocking procaspase-9 activation.* Oncogene, 2004. **23**(22): p. 3915-31.

97. Gibson, L., et al., *bcl-w, a novel member of the bcl-2 family, promotes cell survival.* Oncogene, 1996. **13**(4): p. 665-75.

98. Kozopas, K.M., et al., *MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3516-20.

99. Lin, E.Y., et al., *Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2.* J Immunol, 1993. **151**(4): p. 1979-88.

100. Das, R., et al., *Identification of a novel Bcl-2 related gene, BRAG-1, in human glioma.* Oncogene, 1996. **12**(5): p. 947-51.

101. Inohara, N., et al., *Diva, a Bcl-2 homologue that binds directly to Apaf-1 and induces BH3-independent cell death.* J Biol Chem, 1998. **273**(49): p. 32479-86.

102. Ke, N., A. Godzik, and J.C. Reed, *Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak.* J Biol Chem, 2001. **276**(16): p. 12481-4.

103. Zhang, H., W. Holzgreve, and C. De Geyter, *Bcl2-L-10, a novel anti-apoptotic member of the Bcl-2 family, blocks apoptosis in the mitochondria death pathway but not in the death receptor pathway.* Hum Mol Genet, 2001. **10**(21): p. 2329-39.

104. Oltvai, Z.N., C.L. Milliman, and S.J. Korsmeyer, *Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death.* Cell, 1993. **74**(4): p. 609-19.

105. Chittenden, T., et al., *Induction of apoptosis by the Bcl-2 homologue Bak.* Nature, 1995. **374**(6524): p. 733-6.

106. Hsu, S.Y., et al., *Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12401-6.

107. Kataoka, T., et al., *Bcl-rambo, a novel Bcl-2 homologue that induces apoptosis via its unique C-terminal extension.* J Biol Chem, 2001. **276**(22): p. 19548-54.

108. Guo, B., A. Godzik, and J.C. Reed, *Bcl-G, a novel pro-apoptotic member of the Bcl-2 family.* J Biol Chem, 2001. **276**(4): p. 2780-5.

109. Antignani, A. and R.J. Youle, *How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane?* Curr Opin Cell Biol, 2006. **18**(6): p. 685-9.

110. Yang, E., et al., *Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death.* Cell, 1995. **80**(2): p. 285-91.

111. Wang, K., et al., *BID: a novel BH3 domain-only death agonist.* Genes Dev, 1996. **10**(22): p. 2859-69.

112. Boyd, J.M., et al., *Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins.* Oncogene, 1995. **11**(9): p. 1921-8.

113. O'Connor, L., et al., *Bim: a novel member of the Bcl-2 family that promotes apoptosis.* Embo J, 1998. **17**(2): p. 384-95.

114. Inohara, N., et al., *harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X(L).* Embo J, 1997. **16**(7): p. 1686-94.

115. Zhang, H., J. Heim, and B. Meyhack, *Novel BNIP1 variants and their interaction with BCL2 family members.* FEBS Lett, 1999. **448**(1): p. 23-7.

116. Yasuda, M., et al., *BNIP3alpha: a human homolog of mitochondrial proapoptotic protein BNIP3.* Cancer Res, 1999. **59**(3): p. 533-7.

117. Nakano, K. and K.H. Vousden, *PUMA, a novel proapoptotic gene, is induced by p53.* Mol Cell, 2001. **7**(3): p. 683-94.

118. Oda, E., et al., *Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis.* Science, 2000. **288**(5468): p. 1053-8.

119. Bae, J., et al., *MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain.* J Biol Chem, 2000. **275**(33): p. 25255-61.

120. Puthalakath, H., et al., *Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis.* Science, 2001. **293**(5536): p. 1829-32.

121. Tan, K.O., et al., *MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains.* J Biol Chem, 2001. **276**(4): p. 2802-7.

122. Fleischer, A., et al., *Proapoptotic activity of ITM2B(s), a BH3-only protein induced upon IL-2-deprivation which interacts with Bcl-2.* Oncogene, 2002. **21**(20): p. 3181-9.

123. Mund, T., et al., *Spike, a novel BH3-only protein, regulates apoptosis at the endoplasmic reticulum.* Faseb J, 2003. **17**(6): p. 696-8.

124. Kim, H., et al., *Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies.* Nat Cell Biol, 2006. **8**(12): p. 1348-58.

125. Willis, S.N. and J.M. Adams, *Life in the balance: how BH3-only proteins induce apoptosis.* Curr Opin Cell Biol, 2005. **17**(6): p. 617-25.

126. Griffiths, G.J., et al., *Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis.* J Cell Biol, 1999. **144**(5): p. 903-14.

127. Gross, A., et al., *Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis.* Embo J, 1998. **17**(14): p. 3878-85.

128. Wolter, K.G., et al., *Movement of Bax from the cytosol to mitochondria during apoptosis.* J Cell Biol, 1997. **139**(5): p. 1281-92.

129. Hsu, Y.T., K.G. Wolter, and R.J. Youle, *Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3668-72.

130. Kuwana, T., et al., *Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane.* Cell, 2002. **111**(3): p. 331-42.

131. Basanez, G., et al., *Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5492-7.

132. Kudla, G., et al., *The destabilization of lipid membranes induced by the C-terminal fragment of caspase 8-cleaved bid is inhibited by the N-terminal fragment.* J Biol Chem, 2000. **275**(30): p. 22713-8.

133. Epand, R.F., et al., *The apoptotic protein tBid promotes leakage by altering membrane curvature.* J Biol Chem, 2002. **277**(36): p. 32632-9.

134. Epand, R.F., et al., *Membrane perturbations induced by the apoptotic Bax protein.* Biochem J, 2002. **367**(Pt 3): p. 849-55.

135. Sugiyama, T., et al., *Activation of mitochondrial voltage-dependent anion channel by apro-apoptotic BH3-only protein Bim.* Oncogene, 2002. **21**(32): p. 4944-56.

136. Marzo, I., et al., *Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis.* Science, 1998. **281**(5385): p. 2027-31.

137. Wei, M.C., et al., *Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death.* Science, 2001. **292**(5517): p. 727-30.

138. Yin, X.M., et al., *Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis.* Nature, 1999. **400**(6747): p. 886-91.

139. Cartron, P.F., et al., *The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA.* Mol Cell, 2004. **16**(5): p. 807-18.

140. Chen, L., et al., *Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function.* Mol Cell, 2005. **17**(3): p. 393-403.

141. Willis, S.N., et al., *Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins.* Genes Dev, 2005. **19**(11): p. 1294-305.

142. Wang, X., et al., *Oestrogen signalling inhibits invasive phenotype by repressing RelB and its target BCL2.* Nat Cell Biol, 2007. **9**(4): p. 470-8.

143. Viatour, P., et al., *NF- kappa B2/p100 induces Bcl-2 expression.* Leukemia, 2003. **17**(7): p. 1349-56.

144. Grad, J.M., X.R. Zeng, and L.H. Boise, *Regulation of Bcl-xL: a little bit of this and a little bit of STAT.* Curr Opin Oncol, 2000. **12**(6): p. 543-9.

145. Le Gouill, S., et al., *Mcl-1 regulation and its role in multiple myeloma.* Cell Cycle, 2004. **3**(10): p. 1259-62.

146. Melino, G., et al., *p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation.* J Biol Chem, 2004. **279**(9): p. 8076-83.

147. Hershko, T. and D. Ginsberg, *Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis.* J Biol Chem, 2004. **279**(10): p. 8627-34.

148. Obexer, P., et al., *FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells.* Cell Death Differ, 2007. **14**(3): p. 534-47.

149. Puthalakath, H., et al., *ER stress triggers apoptosis by activating BH3-only protein Bim.* Cell, 2007. **129**(7): p. 1337-49.

150. Marani, M., et al., *Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis.* Mol Cell Biol, 2002. **22**(11): p. 3577-89.

151. Renshaw, S.A., et al., *Three novel Bid proteins generated by alternative splicing of the human Bid gene.* J Biol Chem, 2004. **279**(4): p. 2846-55.

152. Schmitt, E., et al., *Characterization of Bax-sigma, a cell death-inducing isoform of Bax.* Biochem Biophys Res Commun, 2000. **270**(3): p. 868-79.

153. Li, H., et al., *Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis.* Cell, 1998. **94**(4): p. 491-501.

154. Zha, J., et al., *Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis.* Science, 2000. **290**(5497): p. 1761-5.

155. She, Q.B., et al., *The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells.* Cancer Cell, 2005. **8**(4): p. 287-97.

156. Yang, H., et al., *The proapoptotic protein Bad binds the amphipathic groove of 14-3-3zeta.* Biochim Biophys Acta, 2001. **1547**(2): p. 313-9.

157. Wang, H.G., et al., *Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD.* Science, 1999. **284**(5412): p. 339-43.

158. Verma, S., L.J. Zhao, and G. Chinnadurai, *Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis.* J Biol Chem, 2001. **276**(7): p. 4671-6.

159. Lei, K. and R.J. Davis, *JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2432-7.

160. Domina, A.M., et al., *MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol.* Oncogene, 2004. **23**(31): p. 5301-15.

161. Yuan, J., et al., *The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme.* Cell, 1993. **75**(4): p. 641-52.

162. Kumar, S. and P.A. Colussi, *Prodomains--adaptors--oligomerization: the pursuit of caspase activation in apoptosis.* Trends Biochem Sci, 1999. **24**(1): p. 1-4.

163. Wilson, K.P., et al., *Structure and mechanism of interleukin-1 beta converting enzyme.* Nature, 1994. **370**(6487): p. 270-5.

164. Walker, N.P., et al., *Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer.* Cell, 1994. **78**(2): p. 343-52.

165. Rotonda, J., et al., *The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis.* Nat Struct Biol, 1996. **3**(7): p. 619-25.

166. Mittl, P.R., et al., *Structure of recombinant human CPP32 in complex with the tetrapeptide acetyl-Asp-Val-Ala-Asp fluoromethyl ketone.* J Biol Chem, 1997. **272**(10): p. 6539-47.

167. Cryns, V. and J. Yuan, *Proteases to die for.* Genes Dev, 1998. **12**(11): p. 1551-70.

168. Alnemri, E.S., *Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases.* J Cell Biochem, 1997. **64**(1): p. 33-42.

169. Zou, H., et al., *Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3.* Cell, 1997. **90**(3): p. 405-13.

170. Zou, H., et al., *An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9.* J Biol Chem, 1999. **274**(17): p. 11549-56.

171. Li, P., et al., *Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.* Cell, 1997. **91**(4): p. 479-89.

172. Cain, K., et al., *Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex.* J Biol Chem, 1999. **274**(32): p. 22686-92.

173. Aravind, L., V.M. Dixit, and E.V. Koonin, *The domains of death: evolution of the apoptosis machinery.* Trends Biochem Sci, 1999. **24**(2): p. 47-53.

174. Tinel, A. and J. Tschopp, *The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress.* Science, 2004. **304**(5672): p. 843-6.

175. Duan, H. and V.M. Dixit, *RAIDD is a new 'death' adaptor molecule.* Nature, 1997. **385**(6611): p. 86-9.

176. Ahmad, M., et al., *CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor-interacting protein RIP.* Cancer Res, 1997. **57**(4): p. 615-9.

177. Locksley, R.M., N. Killeen, and M.J. Lenardo, *The TNF and TNF receptor superfamilies: integrating mammalian biology.* Cell, 2001. **104**(4): p. 487-501.

178. Salvesen, G.S. and V.M. Dixit, *Caspase activation: the induced-proximity model.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 10964-7.

179. Nagata, S., *Apoptosis by death factor.* Cell, 1997. **88**(3): p. 355-65.

180. Kischkel, F.C., et al., *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor.* Embo J, 1995. **14**(22): p. 5579-88.

181. Budihardjo, I., et al., *Biochemical pathways of caspase activation during apoptosis.* Annu Rev Cell Dev Biol, 1999. **15**: p. 269-90.

182. Sakahira, H., M. Enari, and S. Nagata, *Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.* Nature, 1998. **391**(6662): p. 96-9.

183. Enari, M., et al., *A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD.* Nature, 1998. **391**(6662): p. 43-50.

184. Soldani, C. and A.I. Scovassi, *Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update.* Apoptosis, 2002. **7**(4): p. 321-8.

185. Casciola-Rosen, L., et al., *Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death.* J Exp Med, 1996. **183**(5): p. 1957-64.

186. Coleman, M.L., et al., *Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I.* Nat Cell Biol, 2001. **3**(4): p. 339-45.

187. Silke, J. and D.L. Vaux, *Two kinds of BIR-containing protein - inhibitors of apoptosis, or required for mitosis.* J Cell Sci, 2001. **114**(Pt 10): p. 1821-7.

188. Deveraux, Q.L. and J.C. Reed, *IAP family proteins--suppressors of apoptosis.* Genes Dev, 1999. **13**(3): p. 239-52.

189. Takahashi, R., et al., *A single BIR domain of XIAP sufficient for inhibiting caspases.* J Biol Chem, 1998. **273**(14): p. 7787-90.

190. Roy, N., et al., *The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases.* Embo J, 1997. **16**(23): p. 6914-25.

191. Hauser, H.P., et al., *A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors.* J Cell Biol, 1998. **141**(6): p. 1415-22.

192. Chen, Z., et al., *A human IAP-family gene, apollon, expressed in human brain cancer cells.* Biochem Biophys Res Commun, 1999. **264**(3): p. 847-54.

193. Huang, H., et al., *The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7.* J Biol Chem, 2000. **275**(35): p. 26661-4.

194. Yang, Y., et al., *Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli.* Science, 2000. **288**(5467): p. 874-7.

195. Lemasters, J.J., *V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis.* Am J Physiol, 1999. **276**(1 Pt 1): p. G1-6.

196. Ekert, P.G., J. Silke, and D.L. Vaux, *Caspase inhibitors.* Cell Death Differ, 1999. **6**(11): p. 1081-6.

197. Kroemer, G. and S.J. Martin, *Caspase-independent cell death.* Nat Med, 2005. **11**(7): p. 725-30.

198. Roninson, I.B., E.V. Broude, and B.D. Chang, *If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells.* Drug Resist Updat, 2001. **4**(5): p. 303-13.

199. Roninson, I.B., *Tumor cell senescence in cancer treatment.* Cancer Res, 2003. **63**(11): p. 2705-15.

200. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

201. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.

202. Yee, D., *Targeting insulin-like growth factor pathways.* Br J Cancer, 2007. **96 Suppl**: p. R7-10.

203. Lewis, N.L., *The platelet-derived growth factor receptor as a therapeutic target.* Curr Oncol Rep, 2007. **9**(2): p. 89-95.

204. Qi, Y., et al., *Cyclin A but not cyclin D1 is essential for c-myc-modulated cell-cycle progression.* J Cell Physiol, 2007. **210**(1): p. 63-71.

205. Yu, Q., M.A. Ciemerych, and P. Sicinski, *Ras and Myc can drive oncogenic cell proliferation through individual D-cyclins.* Oncogene, 2005. **24**(47): p. 7114-9.

206. Medema, R.H. and J.L. Bos, *The role of p21ras in receptor tyrosine kinase signaling.* Crit Rev Oncog, 1993. **4**(6): p. 615-61.

207. Yarden, Y. and A. Ullrich, *Growth factor receptor tyrosine kinases.* Annu Rev Biochem, 1988. **57**: p. 443-78.

208. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.

209. Keski-Oja, J., et al., *Transforming growth factors and control of neoplastic cell growth.* J Cell Biochem, 1987. **33**(2): p. 95-107.

210. Leder, P., et al., *Translocations among antibody genes in human cancer.* Science, 1983. **222**(4625): p. 765-71.

211. Arvanitis, C. and D.W. Felsher, *Conditionally MYC: insights from novel transgenic models.* Cancer Lett, 2005. **226**(2): p. 95-9.

212. Leevers, S.J., B. Vanhaesebroeck, and M.D. Waterfield, *Signalling through phosphoinositide 3-kinases: the lipids take centre stage.* Curr Opin Cell Biol, 1999. **11**(2): p. 219-25.

213. Millward, T.A., S. Zolnierowicz, and B.A. Hemmings, *Regulation of protein kinase cascades by protein phosphatase 2A.* Trends Biochem Sci, 1999. **24**(5): p. 186-91.

214. Sen, P., et al., *Involvement of the Akt/PKB signaling pathway with disease processes.* Mol Cell Biochem, 2003. **253**(1-2): p. 241-6.

215. Dutcher, J.P., *Mammalian target of rapamycin inhibition.* Clin Cancer Res, 2004. **10**(18 Pt 2): p. 6382S-7S.

216. Dutcher, J.P., *Mammalian target of rapamycin (mTOR) Inhibitors.* Curr Oncol Rep, 2004. **6**(2): p. 111-5.

217. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-41.

218. Madrid, L.V., et al., *Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB.* Mol Cell Biol, 2000. **20**(5): p. 1626-38.

219. Khwaja, A., *Akt is more than just a Bad kinase.* Nature, 1999. **401**(6748): p. 33-4.

220. Buss, H., et al., *Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription.* J Biol Chem, 2004. **279**(53): p. 55633-43.

221. Hoffmann, A., et al., *The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation.* Science, 2002. **298**(5596): p. 1241-5.

222. Schmitz, M.L., M.A. dos Santos Silva, and P.A. Baeuerle, *Transactivation domain 2 (TA2) of p65 NF-kappa B. Similarity to TA1 and phorbol ester-stimulated activity and phosphorylation in intact cells.* J Biol Chem, 1995. **270**(26): p. 15576-84.

223. Chen, L.F. and W.C. Greene, *Shaping the nuclear action of NF-kappaB.* Nat Rev Mol Cell Biol, 2004. **5**(5): p. 392-401.

224. Mordmuller, B., et al., *Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism.* EMBO Rep, 2003. **4**(1): p. 82-7.

225. Lessard, L., et al., *NF-kappaB2 processing and p52 nuclear accumulation after androgenic stimulation of LNCaP prostate cancer cells.* Cell Signal, 2007. **19**(5): p. 1093-100.

226. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.

227. Gutkind, J.S., *Cell growth control by G protein-coupled receptors: from signal transduction to signal integration.* Oncogene, 1998. **17**(11 Reviews): p. 1331-42.

228. Culig, Z., et al., *Interleukin-6 regulation of prostate cancer cell growth.* J Cell Biochem, 2005. **95**(3): p. 497-505.

229. Russell, N.H., *Autocrine growth factors and leukaemic haemopoiesis.* Blood Rev, 1992. **6**(3): p. 149-56.

230. Wang, Y., et al., *Receptors for interleukin-3 (IL-3) and growth hormone mediate an IL-6-type transcriptional induction in the presence of JAK2 or STAT3.* Blood, 1995. **86**(5): p. 1671-9.

231. Matsuda, T. and T. Hirano, *Association of p72 tyrosine kinase with Stat factors and its activation by interleukin-3, interleukin-6, and granulocyte colony-stimulating factor.* Blood, 1994. **83**(12): p. 3457-61.

232. Leclercq, G., et al., *Estrogen receptors in human breast cancer.* Eur J Cancer, 1973. **9**(9): p. 665-73.

233. Dyson, N., et al., *The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product.* Science, 1989. **243**(4893): p. 934-7.

234. Peeper, D.S. and A. Zantema, *Adenovirus-E1A proteins transform cells by sequestering regulatory proteins.* Mol Biol Rep, 1993. **17**(3): p. 197-207.

235. Ravitz, M.J. and C.E. Wenner, *Cyclin-dependent kinase regulation during G1 phase and cell cycle regulation by TGF-beta.* Adv Cancer Res, 1997. **71**: p. 165-207.

236. Nicolas, F.J. and C.S. Hill, *Attenuation of the TGF-beta-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-beta-induced growth arrest.* Oncogene, 2003. **22**(24): p. 3698-711.

237. Kamer, I., et al., *Proapoptotic BID is an ATM effector in the DNA-damage response.* Cell, 2005. **122**(4): p. 593-603.

238. Rotman, G. and Y. Shiloh, *ATM: a mediator of multiple responses to genotoxic stress.* Oncogene, 1999. **18**(45): p. 6135-44.

239. Miyashita, T., et al., *Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo.* Oncogene, 1994. **9**(6): p. 1799-805.

240. Hicks, G.G., et al., *Mutant p53 tumor suppressor alleles release ras-induced cell cycle growth arrest.* Mol Cell Biol, 1991. **11**(3): p. 1344-52.

241. Harris, C.C., *p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective.* Carcinogenesis, 1996. **17**(6): p. 1187-98.

242. Toledo, F. and G.M. Wahl, *Regulating the p53 pathway: in vitro hypotheses, in vivo veritas.* Nat Rev Cancer, 2006. **6**(12): p. 909-23.

243. Kim, W.Y. and N.E. Sharpless, *The regulation of INK4/ARF in cancer and aging.* Cell, 2006. **127**(2): p. 265-75.

244. Wang, C.Y., et al., *NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis.* Mol Cell Biol, 1999. **19**(9): p. 5923-9.

245. Stehlik, C., et al., *Cytokine induced expression of porcine inhibitor of apoptosis protein (iap) family member is regulated by NF-kappa B.* Biochem Biophys Res Commun, 1998. **243**(3): p. 827-32.

246. Wang, C.Y., et al., *NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation.* Science, 1998. **281**(5383): p. 1680-3.

247. Korsmeyer, S.J., *Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes.* Annu Rev Immunol, 1992. **10**: p. 785-807.

248. Cory, S., D.C. Huang, and J.M. Adams, *The Bcl-2 family: roles in cell survival and oncogenesis.* Oncogene, 2003. **22**(53): p. 8590-607.

249. Knudson, C.M., et al., *Bax accelerates tumorigenesis in p53-deficient mice.* Cancer Res, 2001. **61**(2): p. 659-65.

250. Egle, A., et al., *Bim is a suppressor of Myc-induced mouse B cell leukemia.* Proc Natl Acad Sci U S A, 2004. **101**(16): p. 6164-9.

251. Eischen, C.M., et al., *Bax loss impairs Myc-induced apoptosis and circumvents the selection of p53 mutations during Myc-mediated lymphomagenesis.* Mol Cell Biol, 2001. **21**(22): p. 7653-62.

252. Zinkel, S.S., et al., *Proapoptotic BID is required for myeloid homeostasis and tumor suppression.* Genes Dev, 2003. **17**(2): p. 229-39.

253. Villunger, A., et al., *p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa.* Science, 2003. **302**(5647): p. 1036-8.

254. Shibue, T., et al., *Integral role of Noxa in p53-mediated apoptotic response.* Genes Dev, 2003. **17**(18): p. 2233-8.

255. Hemann, M.T., et al., *Suppression of tumorigenesis by the p53 target PUMA.* Proc Natl Acad Sci U S A, 2004. **101**(25): p. 9333-8.

256. Dunham, M.A., et al., *Telomere maintenance by recombination in human cells.* Nat Genet, 2000. **26**(4): p. 447-50.

257. Liu, D., et al., *Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins.* J Biol Chem, 2004. **279**(49): p. 51338-42.

258. Chan, S.W. and E.H. Blackburn, *New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin.* Oncogene, 2002. **21**(4): p. 553-63.

259. Collins, K. and J.R. Mitchell, *Telomerase in the human organism.* Oncogene, 2002. **21**(4): p. 564-79.

260. Muntoni, A. and R.R. Reddel, *The first molecular details of ALT in human tumor cells.* Hum Mol Genet, 2005. **14 Spec No. 2**: p. R191-6.

261. Veikkola, T. and K. Alitalo, *VEGFs, receptors and angiogenesis.* Semin Cancer Biol, 1999. **9**(3): p. 211-20.

262. Choi, K.S., et al., *Hypoxia-induced angiogenesis during carcinogenesis.* J Biochem Mol Biol, 2003. **36**(1): p. 120-7.

263. Maxwell, P.H., et al., *The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.* Nature, 1999. **399**(6733): p. 271-5.

264. Kim, W.Y. and W.G. Kaelin, *Role of VHL gene mutation in human cancer.* J Clin Oncol, 2004. **22**(24): p. 4991-5004.

265. Hopfl, G., et al., *Rescue of hypoxia-inducible factor-1alpha-deficient tumor growth by wild-type cells is independent of vascular endothelial growth factor.* Cancer Res, 2002. **62**(10): p. 2962-70.

266. Hudson, C.C., et al., *Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin.* Mol Cell Biol, 2002. **22**(20): p. 7004-14.

267. Bernhard, E.J., S.B. Gruber, and R.J. Muschel, *Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells.* Proc Natl Acad Sci U S A, 1994. **91**(10): p. 4293-7.

268. Naylor, M.S., et al., *Expression and activity of MMPS and their regulators in ovarian cancer.* Int J Cancer, 1994. **58**(1): p. 50-6.

269. Christofori, G. and H. Semb, *The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene.* Trends Biochem Sci, 1999. **24**(2): p. 73-6.

270. Partin, A.C., DS, *The Molecular Biology, Endocrinology and Physiology of the Prostate and Seminal Vesicles*, in *Campbell's Urology*, P.R. Walsh, AB; Vaughan, ED Jr.; Wein, AJ, Editor. 1998, Sauders: Philadelphia. p. 1381.

271. Brooks, J., *Anatomy of the Lower Urinary Tract and Male Genitalia*, in *Campbell's Urology*, P.R. Walsh, AB; Vaughan, ED Jr.; Wein, AJ, Editor. 1998, Sauders: Philadelphia. p. 89.

272. McNeal, J.E., *The zonal anatomy of the prostate.* Prostate, 1981. **2**(1): p. 35-49.

273. Aumuller, G. and J. Seitz, *Protein secretion and secretory processes in male accessory sex glands.* Int Rev Cytol, 1990. **121**: p. 127-231.

274. Mann, T., *Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction.* J Reprod Fertil, 1974. **37**(1): p. 179-88.

275. Lam, J.S. and R.E. Reiter, *Stem cells in prostate and prostate cancer development.* Urol Oncol, 2006. **24**(2): p. 131-40.

276. Rizzo, S., G. Attard, and D.L. Hudson, *Prostate epithelial stem cells.* Cell Prolif, 2005. **38**(6): p. 363-74.

277. Bonkhoff, H., *Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status.* Prostate Suppl, 1998. **8**: p. 18-22.

278. Hayward, S.W., M.A. Rosen, and G.R. Cunha, *Stromal-epithelial interactions in the normal and neoplastic prostate.* Br J Urol, 1997. **79 Suppl 2**: p. 18-26.

279. Chatterjee, B., *The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer.* Mol Cell Biochem, 2003. **253**(1-2): p. 89-101.

280. Schalken, J.A. and G. van Leenders, *Cellular and molecular biology of the prostate: stem cell biology.* Urology, 2003. **62**(5 Suppl 1): p. 11-20.

281. Kitay, J.I., N.H. Swygert, and M.D. Coyne, *Effects of hypophysectomy and administration of cortisone or ACTH on adrenal 5-alpha-reductase activity and steroid production.* Endocrinology, 1971. **89**(2): p. 432-8.

282. Eik-Nes, K.B., *Production and secretion of 5alpha-reduced testosterone (DHT) by male reproductive organs.* J Steroid Biochem, 1975. **6**(3-4): p. 337-9.

283. Corbin, A. and F.J. Bex, *Reproductive pharmacology of LHRH and agonists in females and males.* Acta Eur Fertil, 1980. **11**(2): p. 113-30.

284. Odell, W.D. and L.N. Parker, *Control of adrenal androgen production.* Endocr Res, 1984. **10**(3-4): p. 617-30.

285. Bieberich, C.J., et al., *Prostate-specific and androgen-dependent expression of a novel homeobox gene.* J Biol Chem, 1996. **271**(50): p. 31779-82.

286. Abdulkadir, S.A., et al., *Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia.* Mol Cell Biol, 2002. **22**(5): p. 1495-503.

287. Donjacour, A.A., A.A. Thomson, and G.R. Cunha, *FGF-10 plays an essential role in the growth of the fetal prostate.* Dev Biol, 2003. **261**(1): p. 39-54.

288. Pu, Y., et al., *Androgen regulation of prostate morphoregulatory gene expression: Fgf10-dependent and -independent pathways.* Endocrinology, 2007. **148**(4): p. 1697-706.

289. McNeal, J.E., *Origin and development of carcinoma in the prostate.* Cancer, 1969. **23**(1): p. 24-34.

290. Powell, W.C., et al., *Mouse strains for prostate tumorigenesis based on genes altered in human prostate cancer.* Curr Drug Targets, 2003. **4**(3): p. 263-79.

291. Canada, C.C.S.N.C.I.o., *Canadian Cancer Statistics*. 2007: Toronto, Canada.

292. Canada, C.C.S.N.C.I.o., *Canadian Cancer Statistics*. 2006, Canadian Cancer Society/ National Cancer Institute of Canada.

293. Bratt, O., *Hereditary prostate cancer: clinical aspects.* J Urol, 2002. **168**(3): p. 906-13.

294. Carpten, J., et al., *Germline mutations in the ribonuclease L gene in families showing linkage with HPC1.* Nat Genet, 2002. **30**(2): p. 181-4.

295. Rebbeck, T.R., et al., *Association of HPC2/ELAC2 genotypes and prostate cancer.* Am J Hum Genet, 2000. **67**(4): p. 1014-9.

296. Bisbal, C. and R.H. Silverman, *Diverse functions of RNase L and implications in pathology.* Biochimie, 2007. **89**(6-7): p. 789-98.

297. Noda, D., et al., *ELAC2, a putative prostate cancer susceptibility gene product, potentiates TGF-beta/Smad-induced growth arrest of prostate cells.* Oncogene, 2006. **25**(41): p. 5591-600.

298. Cybulski, C., et al., *A novel founder CHEK2 mutation is associated with increased prostate cancer risk.* Cancer Res, 2004. **64**(8): p. 2677-9.

299. Cybulski, C., et al., *NBS1 is a prostate cancer susceptibility gene.* Cancer Res, 2004. **64**(4): p. 1215-9.

300. Hughes, C., et al., *Molecular pathology of prostate cancer.* J Clin Pathol, 2005. **58**(7): p. 673-84.

301. Schulz, W.A., M. Burchardt, and M.V. Cronauer, *Molecular biology of prostate cancer.* Mol Hum Reprod, 2003. **9**(8): p. 437-48.

302. Nam, R.K., et al., *V89L polymorphism of type-2, 5-alpha reductase enzyme gene predicts prostate cancer presence and progression.* Urology, 2001. **57**(1): p. 199-204.

303. Fraser, M.L., A.H. Lee, and C.W. Binns, *Lycopene and prostate cancer: emerging evidence.* Expert Rev Anticancer Ther, 2005. **5**(5): p. 847-54.

304. Vogt, T.M., et al., *Serum lycopene, other serum carotenoids, and risk of prostate cancer in US Blacks and Whites.* Am J Epidemiol, 2002. **155**(11): p. 1023-32.

305. Giovannucci, E., *The epidemiology of vitamin D and cancer incidence and mortality: a review (United States).* Cancer Causes Control, 2005. **16**(2): p. 83-95.

306. Vlajinac, H.D., et al., *Diet and prostate cancer: a case-control study.* Eur J Cancer, 1997. **33**(1): p. 101-7.

307. Yoshizawa, K., et al., *Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer.* J Natl Cancer Inst, 1998. **90**(16): p. 1219-24.

308. Cohen, J.H., A.R. Kristal, and J.L. Stanford, *Fruit and vegetable intakes and prostate cancer risk.* J Natl Cancer Inst, 2000. **92**(1): p. 61-8.

309. Schoonen, W.M., et al., *Alcohol consumption and risk of prostate cancer in middle-aged men.* Int J Cancer, 2005. **113**(1): p. 133-40.

310. Jian, L., et al., *Protective effect of green tea against prostate cancer: a case-control study in southeast China.* Int J Cancer, 2004. **108**(1): p. 130-5.

311. Bemis, D.L., A.E. Katz, and R. Buttyan, *Clinical trials of natural products as chemopreventive agents for prostate cancer.* Expert Opin Investig Drugs, 2006. **15**(10): p. 1191-200.

312. Lee, M.M., et al., *Soy and isoflavone consumption in relation to prostate cancer risk in China.* Cancer Epidemiol Biomarkers Prev, 2003. **12**(7): p. 665-8.

313. Chan, J.M., P.H. Gann, and E.L. Giovannucci, *Role of diet in prostate cancer development and progression.* J Clin Oncol, 2005. **23**(32): p. 8152-60.

314. Crawford, E.D., *Epidemiology of prostate cancer.* Urology, 2003. **62**(6 Suppl 1): p. 3-12.

315. Melamed, J., J.M. Einhorn, and M.M. Ittmann, *Allelic loss on chromosome 13q in human prostate carcinoma.* Clin Cancer Res, 1997. **3**(10): p. 1867-72.

316. Jarrard, D.F., et al., *Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer.* Genes Chromosomes Cancer, 1997. **19**(2): p. 90-6.

317. Cher, M.L., et al., *Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping.* Cancer Res, 1996. **56**(13): p. 3091-102.

318. Sato, K., et al., *Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma.* J Natl Cancer Inst, 1999. **91**(18): p. 1574-80.

319. Cooney, K.A., et al., *Identification and characterization of proximal 6q deletions in prostate cancer.* Cancer Res, 1996. **56**(18): p. 4150-3.

320. Trapman, J., et al., *Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostate cancer.* Cancer Res, 1994. **54**(23): p. 6061-4.

321. Emmert-Buck, M.R., et al., *Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia.* Cancer Res, 1995. **55**(14): p. 2959-62.

322. Vocke, C.D., et al., *Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21.* Cancer Res, 1996. **56**(10): p. 2411-6.

323. Bova, G.S., et al., *Physical mapping of chromosome 8p22 markers and their homozygous deletion in a metastatic prostate cancer.* Genomics, 1996. **35**(1): p. 46-54.

324. Bova, G.S., et al., *Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer.* Cancer Res, 1993. **53**(17): p. 3869-73.

325. Cabeza-Arvelaiz, Y., et al., *LAPSER1: a novel candidate tumor suppressor gene from 10q24.3.* Oncogene, 2001. **20**(46): p. 6707-17.

326. Ishii, H., et al., *The FEZ1 gene at chromosome 8p22 encodes a leucine-zipper protein, and its expression is altered in multiple human tumors.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3928-33.

327. Narla, G., et al., *KLF6, a candidate tumor suppressor gene mutated in prostate cancer.* Science, 2001. **294**(5551): p. 2563-6.

328. Fukuhara, H., et al., *Functional evidence for the presence of tumor suppressor gene on chromosome 10p15 in human prostate cancers.* Oncogene, 2001. **20**(3): p. 314-9.

329. Feilotter, H.E., et al., *Analysis of PTEN and the 10q23 region in primary prostate carcinomas.* Oncogene, 1998. **16**(13): p. 1743-8.

330. Rubin, M.A., et al., *10q23.3 loss of heterozygosity is higher in lymph node-positive (pT2-3,N+) versus lymph node-negative (pT2-3,N0) prostate cancer.* Hum Pathol, 2000. **31**(4): p. 504-8.

331. Latil, A., et al., *CHC1-L, a candidate gene for prostate carcinogenesis at 13q14.2, is frequently affected by loss of heterozygosity and underexpressed in human prostate cancer.* Int J Cancer, 2002. **99**(5): p. 689-96.

332. Carter, B.S., et al., *Allelic loss of chromosomes 16q and 10q in human prostate cancer.* Proc Natl Acad Sci U S A, 1990. **87**(22): p. 8751-5.

333. Cooney, K.A., et al., *Distinct regions of allelic loss on 13q in prostate cancer.* Cancer Res, 1996. **56**(5): p. 1142-5.

334. Brooks, J.D., et al., *An uncertain role for p53 gene alterations in human prostate cancers.* Cancer Res, 1996. **56**(16): p. 3814-22.

335. Wang, S., et al., *Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer.* Cancer Cell, 2003. **4**(3): p. 209-21.

336. Ge, K., et al., *Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma.* Int J Cancer, 2000. **86**(2): p. 155-61.

337. Alers, J.C., et al., *Interphase cytogenetics of prostatic adenocarcinoma and precursor lesions: analysis of 25 radical prostatectomies and 17 adjacent prostatic intraepithelial neoplasias.* Genes Chromosomes Cancer, 1995. **12**(4): p. 241-50.

338. Tsuchiya, N., et al., *Mapping and gene expression profile of the minimally overrepresented 8q24 region in prostate cancer.* Am J Pathol, 2002. **160**(5): p. 1799-806.

339. Kaltz-Wittmer, C., et al., *FISH analysis of gene aberrations (MYC, CCND1, ERBB2, RB, and AR) in advanced prostatic carcinomas before and after androgen deprivation therapy.* Lab Invest, 2000. **80**(9): p. 1455-64.

340. Cher, M.L., et al., *Comparative genomic hybridization, allelic imbalance, and fluorescence in situ hybridization on chromosome 8 in prostate cancer.* Genes Chromosomes Cancer, 1994. **11**(3): p. 153-62.

341. Ellwood-Yen, K., et al., *Myc-driven murine prostate cancer shares molecular features with human prostate tumors.* Cancer Cell, 2003. **4**(3): p. 223-38.

342. Tanaka, M., et al., *Nkx3.1, a murine homolog of Ddrosophila bagpipe, regulates epithelial ductal branching and proliferation of the prostate and palatine glands.* Dev Dyn, 2000. **219**(2): p. 248-60.

343. Lei, Q., et al., *NKX3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss.* Cancer Cell, 2006. **9**(5): p. 367-78.

344. Tomlins, S.A., et al., *Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer.* Science, 2005. **310**(5748): p. 644-8.

345. Stanbrough, M., et al., *Prostatic intraepithelial neoplasia in mice expressing an androgen receptor transgene in prostate epithelium.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10823-8.

346. Huggins, C. and C.V. Hodges, *Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate.* CA Cancer J Clin, 1972. **22**(4): p. 232-40.

347. Shang, Y., M. Myers, and M. Brown, *Formation of the androgen receptor transcription complex.* Mol Cell, 2002. **9**(3): p. 601-10.

348. Simental, J.A., et al., *Transcriptional activation and nuclear targeting signals of the human androgen receptor.* J Biol Chem, 1991. **266**(1): p. 510-8.

349. Hsiao, P.W., et al., *The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator.* J Biol Chem, 1999. **274**(29): p. 20229-34.

350. Yeh, S. and C. Chang, *Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells.* Proc Natl Acad Sci U S A, 1996. **93**(11): p. 5517-21.

351. Hsiao, P.W. and C. Chang, *Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells.* J Biol Chem, 1999. **274**(32): p. 22373-9.

352. Wang, X., et al., *Identification and characterization of a novel androgen receptor coregulator ARA267-alpha in prostate cancer cells.* J Biol Chem, 2001. **276**(44): p. 40417-23.

353. Alen, P., et al., *The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription.* Mol Cell Biol, 1999. **19**(9): p. 6085-97.

354. Aarnisalo, P., J.J. Palvimo, and O.A. Janne, *CREB-binding protein in androgen receptor-mediated signaling.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2122-7.

355. Lee, D.K., H.O. Duan, and C. Chang, *From androgen receptor to the general transcription factor TFIIH. Identification of cdk activating kinase (CAK) as an androgen receptor NH(2)-terminal associated coactivator.* J Biol Chem, 2000. **275**(13): p. 9308-13.

356. Yamamoto, A., et al., *Cyclin E as a coactivator of the androgen receptor.* J Cell Biol, 2000. **150**(4): p. 873-80.

357. Yeh, S., et al., *Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells.* Biochem Biophys Res Commun, 1998. **248**(2): p. 361-7.

358. Park, J.J., et al., *Breast cancer susceptibility gene 1 (BRCAI) is a coactivator of the androgen receptor.* Cancer Res, 2000. **60**(21): p. 5946-9.

359. Kupfer, S.R., et al., *Receptor accessory factor enhances specific DNA binding of androgen and glucocorticoid receptors.* J Biol Chem, 1993. **268**(23): p. 17519-27.

360. Koshy, B., et al., *Spinocerebellar ataxia type-1 and spinobulbar muscular atrophy gene products interact with glyceraldehyde-3-phosphate dehydrogenase.* Hum Mol Genet, 1996. **5**(9): p. 1311-8.

361. Lu, M.L., et al., *Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation.* J Biol Chem, 2001. **276**(16): p. 13442-51.

362. Beauchemin, A.M., et al., *Cytochrome c oxidase subunit Vb interacts with human androgen receptor: a potential mechanism for neurotoxicity in spinobulbar muscular atrophy.* Brain Res Bull, 2001. **56**(3-4): p. 285-97.

363. Buchanan, G., et al., *Contribution of the androgen receptor to prostate cancer predisposition and progression.* Cancer Metastasis Rev, 2001. **20**(3-4): p. 207-23.

364. Gelmann, E.P., *Molecular biology of the androgen receptor.* J Clin Oncol, 2002. **20**(13): p. 3001-15.

365. Zhou, Z.X., et al., *A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences.* J Biol Chem, 1994. **269**(18): p. 13115-23.

366. Hard, T., et al., *Solution structure of the glucocorticoid receptor DNA-binding domain.* Science, 1990. **249**(4965): p. 157-60.

367. Bubulya, A., et al., *c-Jun can mediate androgen receptor-induced transactivation.* J Biol Chem, 1996. **271**(40): p. 24583-9.

368. Oettgen, P., et al., *PDEF, a novel prostate epithelium-specific ets transcription factor, interacts with the androgen receptor and activates prostate-specific antigen gene expression.* J Biol Chem, 2000. **275**(2): p. 1216-25.

369. Jorgensen, J.S. and J.H. Nilson, *AR suppresses transcription of the alpha glycoprotein hormone subunit gene through protein-protein interactions*

with cJun and activation transcription factor 2. Mol Endocrinol, 2001. **15**(9): p. 1496-504.

370. Palvimo, J.J., et al., *Mutual transcriptional interference between RelA and androgen receptor.* J Biol Chem, 1996. **271**(39): p. 24151-6.
371. Dedhar, S., et al., *Inhibition of nuclear hormone receptor activity by calreticulin.* Nature, 1994. **367**(6462): p. 480-3.
372. Knudsen, K.E., W.K. Cavenee, and K.C. Arden, *D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability.* Cancer Res, 1999. **59**(10): p. 2297-301.
373. Kang, H.Y., et al., *Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor.* J Biol Chem, 1999. **274**(13): p. 8570-6.
374. Fujimoto, N., et al., *Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate.* J Biol Chem, 1999. **274**(12): p. 8316-21.
375. Blanco, J.C., et al., *The histone acetylase PCAF is a nuclear receptor coactivator.* Genes Dev, 1998. **12**(11): p. 1638-51.
376. Song, L.N., et al., *Beta-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription.* Mol Cell Biol, 2003. **23**(5): p. 1674-87.
377. Kang, H.Y., et al., *From transforming growth factor-beta signaling to androgen action: identification of Smad3 as an androgen receptor coregulator in prostate cancer cells.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3018-23.
378. Shenk, J.L., et al., *p53 represses androgen-induced transactivation of prostate-specific antigen by disrupting hAR amino- to carboxyl-terminal interaction.* J Biol Chem, 2001. **276**(42): p. 38472-9.
379. MacLean, H.E., G.L. Warne, and J.D. Zajac, *Localization of functional domains in the androgen receptor.* J Steroid Biochem Mol Biol, 1997. **62**(4): p. 233-42.

380. Smith, D.F., *Dynamics of heat shock protein 90-progesterone receptor binding and the disactivation loop model for steroid receptor complexes.* Mol Endocrinol, 1993. **7**(11): p. 1418-29.

381. Smith, D.F. and D.O. Toft, *Steroid receptors and their associated proteins.* Mol Endocrinol, 1993. **7**(1): p. 4-11.

382. Rao, J., et al., *Functional interaction of human Cdc37 with the androgen receptor but not with the glucocorticoid receptor.* J Biol Chem, 2001. **276**(8): p. 5814-20.

383. Lin, H.K., et al., *Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7200-5.

384. Lin, H.K., et al., *Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase.* Embo J, 2002. **21**(15): p. 4037-48.

385. Ikonen, T., et al., *Stimulation of androgen-regulated transactivation by modulators of protein phosphorylation.* Endocrinology, 1994. **135**(4): p. 1359-66.

386. Guo, Z., et al., *Regulation of androgen receptor activity by tyrosine phosphorylation.* Cancer Cell, 2006. **10**(4): p. 309-19.

387. Gioeli, D., et al., *Stress kinase signaling regulates androgen receptor phosphorylation, transcription, and localization.* Mol Endocrinol, 2006. **20**(3): p. 503-15.

388. Gioeli, D., et al., *Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites.* J Biol Chem, 2002. **277**(32): p. 29304-14.

389. Zhou, Z.X., J.A. Kemppainen, and E.M. Wilson, *Identification of three proline-directed phosphorylation sites in the human androgen receptor.* Mol Endocrinol, 1995. **9**(5): p. 605-15.

390. Heinlein, C.A. and C. Chang, *Androgen receptor in prostate cancer.* Endocr Rev, 2004. **25**(2): p. 276-308.

391. Nolte, R.T., et al., *Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma.* Nature, 1998. **395**(6698): p. 137-43.

392. Westin, S., et al., *Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators.* Nature, 1998. **395**(6698): p. 199-202.

393. Hendriksen, P.J., et al., *Evolution of the androgen receptor pathway during progression of prostate cancer.* Cancer Res, 2006. **66**(10): p. 5012-20.

394. Velasco, A.M., et al., *Identification and validation of novel androgen-regulated genes in prostate cancer.* Endocrinology, 2004. **145**(8): p. 3913-24.

395. Bruckheimer, E.M., et al., *Regulation of Bcl-2 expression by dihydrotestosterone in hormone sensitive LNCaP-FGC prostate cancer cells.* J Urol, 2003. **169**(4): p. 1553-7.

396. Kousteni, S., et al., *Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.* Cell, 2001. **104**(5): p. 719-30.

397. Bonaccorsi, L., et al., *Altered endocytosis of epidermal growth factor receptor in androgen receptor positive prostate cancer cell lines.* J Mol Endocrinol, 2007. **38**(1-2): p. 51-66.

398. Wang, Y., et al., *Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival.* Mol Cell Biol, 2002. **22**(20): p. 7279-90.

399. Gingrich, J.R., et al., *Metastatic prostate cancer in a transgenic mouse.* Cancer Res, 1996. **56**(18): p. 4096-102.

400. Kasper, S. and J.A. Smith, Jr., *Genetically modified mice and their use in developing therapeutic strategies for prostate cancer.* J Urol, 2004. **172**(1): p. 12-9.

401. Stone, K.R., et al., *Isolation of a human prostate carcinoma cell line (DU 145).* Int J Cancer, 1978. **21**(3): p. 274-81.

402. van Bokhoven, A., et al., *Spectral karyotype (SKY) analysis of human prostate carcinoma cell lines.* Prostate, 2003. **57**(3): p. 226-44.

403. van Bokhoven, A., et al., *Molecular characterization of human prostate carcinoma cell lines.* Prostate, 2003. **57**(3): p. 205-25.

404. Sherwood, E.R., et al., *Differential cytokeratin expression in normal, hyperplastic and malignant epithelial cells from human prostate.* J Urol, 1990. **143**(1): p. 167-71.

405. Billstrom, A., et al., *Differential expression of uPA in an aggressive (DU 145) and a nonaggressive (1013L) human prostate cancer xenograft.* Prostate, 1995. **26**(2): p. 94-104.

406. Sobel, R.E. and M.D. Sadar, *Cell lines used in prostate cancer research: a compendium of old and new lines--part 1.* J Urol, 2005. **173**(2): p. 342-59.

407. Kaighn, M.E., et al., *Establishment and characterization of a human prostatic carcinoma cell line (PC-3).* Invest Urol, 1979. **17**(1): p. 16-23.

408. Sramkoski, R.M., et al., *A new human prostate carcinoma cell line, 22Rv1.* In Vitro Cell Dev Biol Anim, 1999. **35**(7): p. 403-9.

409. Tepper, C.G., et al., *Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line.* Cancer Res, 2002. **62**(22): p. 6606-14.

410. Kovar, J.L., et al., *Hyaluronidase expression induces prostate tumor metastasis in an orthotopic mouse model.* Am J Pathol, 2006. **169**(4): p. 1415-26.

411. Horoszewicz, J.S., et al., *The LNCaP cell line--a new model for studies on human prostatic carcinoma.* Prog Clin Biol Res, 1980. **37**: p. 115-32.

412. Veldscholte, J., et al., *A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens.* Biochem Biophys Res Commun, 1990. **173**(2): p. 534-40.

413. Thalmann, G.N., et al., *Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer.* Cancer Res, 1994. **54**(10): p. 2577-81.

414. Stephenson, R.A., et al., *Metastatic model for human prostate cancer using orthotopic implantation in nude mice.* J Natl Cancer Inst, 1992. **84**(12): p. 951-7.

415. Bello, D., et al., *Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18.* Carcinogenesis, 1997. **18**(6): p. 1215-23.

416. Achanzar, W.E., et al., *Cadmium-induced malignant transformation of human prostate epithelial cells.* Cancer Res, 2001. **61**(2): p. 455-8.

417. Webber, M.M., et al., *Human cell lines as an in vitro/in vivo model for prostate carcinogenesis and progression.* Prostate, 2001. **47**(1): p. 1-13.

418. Cooperberg, M.R., J.W. Moul, and P.R. Carroll, *The changing face of prostate cancer.* J Clin Oncol, 2005. **23**(32): p. 8146-51.

419. Young, C.Y., et al., *Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP.* Cancer Res, 1991. **51**(14): p. 3748-52.

420. Delongchamps, N.B., A. Singh, and G.P. Haas, *The role of prevalence in the diagnosis of prostate cancer.* Cancer Control, 2006. **13**(3): p. 158-68.

421. Tenke, P., et al., *Prostate cancer screening.* Recent Results Cancer Res, 2007. **175**: p. 65-81.

422. Anast, J.W., G.L. Andriole, and R.L. Grubb, 2nd, *Managing the local complications of locally advanced prostate cancer.* Curr Urol Rep, 2007. **8**(3): p. 211-6.

423. Puppo, P., *Long-term effects on BPH of medical and instrumental therapies.* Eur Urol, 2001. **39 Suppl 6**: p. 2-6.

424. Gleason, D.F., *Classification of prostatic carcinomas.* Cancer Chemother Rep, 1966. **50**(3): p. 125-8.

425. Epstein, J.I., et al., *Update on the Gleason grading system for prostate cancer: results of an international consensus conference of urologic pathologists.* Adv Anat Pathol, 2006. **13**(1): p. 57-9.

426. Kupelian, P., et al., *Correlation of clinical and pathologic factors with rising prostate-specific antigen profiles after radical prostatectomy alone for clinically localized prostate cancer.* Urology, 1996. **48**(2): p. 249-60.

427. Abrahams, N.A., et al., *Distinguishing atrophy and high-grade prostatic intraepithelial neoplasia from prostatic adenocarcinoma with and without previous adjuvant hormone therapy with the aid of cytokeratin 5/6.* Am J Clin Pathol, 2003. **120**(3): p. 368-76.

428. Wojno, K.J. and J.I. Epstein, *The utility of basal cell-specific anti-cytokeratin antibody (34 beta E12) in the diagnosis of prostate cancer. A review of 228 cases.* Am J Surg Pathol, 1995. **19**(3): p. 251-60.

429. Sooriakumaran, P., S.J. Khaksar, and J. Shah, *Management of prostate cancer. Part 2: localized and locally advanced disease.* Expert Rev Anticancer Ther, 2006. **6**(4): p. 595-603.

430. Stephenson, A.J., et al., *Defining biochemical recurrence of prostate cancer after radical prostatectomy: a proposal for a standardized definition.* J Clin Oncol, 2006. **24**(24): p. 3973-8.

431. Freedland, S.J., et al., *Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy.* Jama, 2005. **294**(4): p. 433-9.

432. Jani, A.B., *Management strategies for locally advanced prostate cancer.* Drugs Aging, 2006. **23**(2): p. 119-29.

433. Sharifi, N., J.L. Gulley, and W.L. Dahut, *Androgen deprivation therapy for prostate cancer.* Jama, 2005. **294**(2): p. 238-44.

434. Masiello, D., et al., *Bicalutamide functions as an androgen receptor antagonist by assembly of a transcriptionally inactive receptor.* J Biol Chem, 2002. **277**(29): p. 26321-6.

435. Klotz, L. and P. Schellhammer, *Combined androgen blockade: the case for bicalutamide.* Clin Prostate Cancer, 2005. **3**(4): p. 215-9.

436. Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer.* Nat Rev Cancer, 2001. **1**(1): p. 34-45.

437. Zhou, J., J. Scholes, and J.T. Hsieh, *Signal transduction targets in androgen-independent prostate cancer.* Cancer Metastasis Rev, 2001. **20**(3-4): p. 351-62.

438. Gao, M., L. Ossowski, and A.C. Ferrari, *Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells and their androgen-independent derivative.* J Cell Physiol, 1999. **179**(3): p. 336-46.

439. Manni, A., et al., *Androgen priming and response to chemotherapy in advanced prostatic cancer.* J Urol, 1986. **136**(6): p. 1242-6.

440. Pienta, K.J. and D. Bradley, *Mechanisms underlying the development of androgen-independent prostate cancer.* Clin Cancer Res, 2006. **12**(6): p. 1665-71.

441. Visakorpi, T., et al., *In vivo amplification of the androgen receptor gene and progression of human prostate cancer.* Nat Genet, 1995. **9**(4): p. 401-6.

442. Rahman, M., H. Miyamoto, and C. Chang, *Androgen receptor coregulators in prostate cancer: mechanisms and clinical implications.* Clin Cancer Res, 2004. **10**(7): p. 2208-19.

443. Linja, M.J., et al., *Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer.* Cancer Res, 2001. **61**(9): p. 3550-5.

444. Culig, Z., et al., *Expression, structure, and function of androgen receptor in advanced prostatic carcinoma.* Prostate, 1998. **35**(1): p. 63-70.

445. Castilla, C., et al., *Bcl-xL is overexpressed in hormone-resistant prostate cancer and promotes survival of LNCaP cells via interaction with proapoptotic Bak.* Endocrinology, 2006. **147**(10): p. 4960-7.

446. Cavarretta, I.T., et al., *The antiapoptotic effect of IL-6 autocrine loop in a cellular model of advanced prostate cancer is mediated by Mcl-1.* Oncogene, 2007. **26**(20): p. 2822-32.

447. Murillo, H., et al., *Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state.* Endocrinology, 2001. **142**(11): p. 4795-805.

448. Debes, J.D. and D.J. Tindall, *Mechanisms of androgen-refractory prostate cancer.* N Engl J Med, 2004. **351**(15): p. 1488-90.

449. Shah, J., S.J. Khaksar, and P. Sooriakumaran, *Management of prostate cancer. Part 3: metastatic disease.* Expert Rev Anticancer Ther, 2006. **6**(5): p. 813-21.

450. Saad, F., J. McKiernan, and J. Eastham, *Rationale for zoledronic acid therapy in men with hormone-sensitive prostate cancer with or without bone metastasis.* Urol Oncol, 2006. **24**(1): p. 4-12.

451. Saad, F., N. Clarke, and M. Colombel, *Natural history and treatment of bone complications in prostate cancer.* Eur Urol, 2006. **49**(3): p. 429-40.

452. Saad, F., *The role of bisphosphonates in the management of prostate cancer.* Curr Oncol Rep, 2006. **8**(3): p. 221-7.

453. Ryan, C.W., et al., *Suppression of bone density loss and bone turnover in patients with hormone-sensitive prostate cancer and receiving zoledronic acid.* BJU Int, 2007. **100**(1): p. 70-5.

454. Coxon, J.P., et al., *Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation.* BJU Int, 2004. **94**(1): p. 164-70.

455. Sooriakumaran, P., *Management of prostate cancer. Part 1: chemoprevention.* Expert Rev Anticancer Ther, 2006. **6**(3): p. 419-25.

456. Thompson, I.M., et al., *Prevention of prostate cancer with finasteride: US/European perspective.* Eur Urol, 2003. **44**(6): p. 650-5.

457. Lowe, J.F. and L.A. Frazee, *Update on prostate cancer chemoprevention.* Pharmacotherapy, 2006. **26**(3): p. 353-9.

458. Klein, E.A., et al., *SELECT: the next prostate cancer prevention trial. Selenum and Vitamin E Cancer Prevention Trial.* J Urol, 2001. **166**(4): p. 1311-5.

459. Hoque, A., et al., *Molecular epidemiologic studies within the Selenium and Vitamin E Cancer Prevention Trial (SELECT).* Cancer Causes Control, 2001. **12**(7): p. 627-33.

460. Thompson, I.M., *Chemoprevention of prostate cancer: agents and study designs.* J Urol, 2007. **178**(3 Pt 2): p. S9-S13.

461. Song, H.H., *Analysis of correlated ROC areas in diagnostic testing.* Biometrics, 1997. **53**(1): p. 370-82.

462. Ghosh, M., J. Crocker, and A. Morris, *Apoptosis in squamous cell carcinoma of the lung: correlation with survival and clinicopathological features.* J Clin Pathol, 2001. **54**(2): p. 111-5.

463. Therneau T, A.E., *An introduction to recursive partitioning using the rpart routine.*, in *Technical Report.* 1997, Section of Biostatistics, Mayo Clinic: Rocester.

464. Su, X. and C.L. Tsai, *Tree-augmented Cox proportional hazards models.* Biostatistics, 2005. **6**(3): p. 486-99.

465. De Rose, A. and A. Pallara, *Survival trees: an alternative non-parametric multivariate technique for life history analysis.* Eur J Popul, 1997. **13**(3): p. 223-41.

466. Altman, D.G. and P. Royston, *What do we mean by validating a prognostic model?* Stat Med, 2000. **19**(4): p. 453-73.

467. Breiman L, F.J., Olshen RA, Stone CJ, *Classification and regression trees.* 1984, California: CRC Press.

468. Liu, X., et al., *Statistical methods for analyzing tissue microarray data.* J Biopharm Stat, 2004. **14**(3): p. 671-85.

469. Theodorescu, D., et al., *p53, bcl-2 and retinoblastoma proteins as long-term prognostic markers in localized carcinoma of the prostate.* J Urol, 1997. **158**(1): p. 131-7.

470. Vesalainen, S. and P. Lipponen, *Expression of retinoblastoma gene (Rb) protein in T12M0 prostatic adenocarcinoma.* J Cancer Res Clin Oncol, 1995. **121**(7): p. 429-33.

471. Tsihlias, J., et al., *Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma.* Cancer Res, 1998. **58**(3): p. 542-8.

472. Cote, R.J., et al., *Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma.* J Natl Cancer Inst, 1998. **90**(12): p. 916-20.

473. Yang, R.M., et al., *Low p27 expression predicts poor disease-free survival in patients with prostate cancer.* J Urol, 1998. **159**(3): p. 941-5.

474. Kuczyk, M., et al., *Predictive value of decreased p27Kip1 protein expression for the recurrence-free and long-term survival of prostate cancer patients.* Br J Cancer, 1999. **81**(6): p. 1052-8.

475. Lee, C.T., et al., *Overexpression of the cyclin-dependent kinase inhibitor p16 is associated with tumor recurrence in human prostate cancer.* Clin Cancer Res, 1999. **5**(5): p. 977-83.

476. Halvorsen, O.J., et al., *Prognostic significance of p16 and CDK4 proteins in localized prostate carcinoma.* Cancer, 2000. **88**(2): p. 416-24.

477. Henshall, S.M., et al., *Overexpression of the cell cycle inhibitor p16INK4A in high-grade prostatic intraepithelial neoplasia predicts early relapse in prostate cancer patients.* Clin Cancer Res, 2001. **7**(3): p. 544-50.

478. Aaltomaa, S., et al., *Prognostic value and expression of p21(waf1/cip1) protein in prostate cancer.* Prostate, 1999. **39**(1): p. 8-15.

479. Matsushima, H., et al., *Immunohistochemical study of p21WAF1 and p53 proteins in prostatic cancer and their prognostic significance.* Hum Pathol, 1998. **29**(8): p. 778-83.

480. Osman, I., et al., *Inactivation of the p53 pathway in prostate cancer: impact on tumor progression.* Clin Cancer Res, 1999. **5**(8): p. 2082-8.

481. Aaltomaa, S., M. Eskelinen, and P. Lipponen, *Expression of cyclin A and D proteins in prostate cancer and their relation to clinopathological variables and patient survival.* Prostate, 1999. **38**(3): p. 175-82.

482. Qian, J., et al., *Loss of p53 and c-myc overrepresentation in stage T(2-3)N(1-3)M(0) prostate cancer are potential markers for cancer progression.* Mod Pathol, 2002. **15**(1): p. 35-44.

483. Borre, M., B. Stausbol-Gron, and J. Overgaard, *p53 accumulation associated with bcl-2, the proliferation marker MIB-1 and survival in patients with prostate cancer subjected to watchful waiting.* J Urol, 2000. **164**(3 Pt 1): p. 716-21.

484. Borre, M., et al., *Immunohistochemical BCL-2 and Ki-67 expression predict survival in prostate cancer patients followed expectantly.* Prostate Cancer Prostatic Dis, 1998. **1**(5): p. 268-275.

485. Borre, M., B. Nerstrom, and J. Overgaard, *The natural history of prostate carcinoma based on a Danish population treated with no intent to cure.* Cancer, 1997. **80**(5): p. 917-28.

486. Quinn, D.I., et al., *Prognostic significance of pathologic features in localized prostate cancer treated with radical prostatectomy: implications for staging systems and predictive models.* J Clin Oncol, 2001. **19**(16): p. 3692-705.

487. Quinn, D.I., et al., *Prognostic significance of preoperative factors in localized prostate carcinoma treated with radical prostatectomy: importance of percentage of biopsies that contain tumor and the presence of biopsy perineural invasion.* Cancer, 2003. **97**(8): p. 1884-93.

488. Quinn, D.I., et al., *Prognostic significance of p53 nuclear accumulation in localized prostate cancer treated with radical prostatectomy.* Cancer Res, 2000. **60**(6): p. 1585-94.

489. Bauer, J.J., et al., *Elevated levels of apoptosis regulator proteins p53 and bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer.* J Urol, 1996. **156**(4): p. 1511-6.

490. Bauer, J.J., et al., *p53 nuclear protein expression is an independent prognostic marker in clinically localized prostate cancer patients undergoing radical prostatectomy.* Clin Cancer Res, 1995. **1**(11): p. 1295-300.

491. Stackhouse, G.B., et al., *p53 and bcl-2 immunohistochemistry in pretreatment prostate needle biopsies to predict recurrence of prostate cancer after radical prostatectomy.* J Urol, 1999. **162**(6): p. 2040-5.

492. Brewster, S.F., et al., *Preoperative p53, bcl-2, CD44 and E-cadherin immunohistochemistry as predictors of biochemical relapse after radical prostatectomy.* J Urol, 1999. **161**(4): p. 1238-43.

493. Scherr, D.S., et al., *BCL-2 and p53 expression in clinically localized prostate cancer predicts response to external beam radiotherapy.* J Urol, 1999. **162**(1): p. 12-6; discussion 16-7.

494. Lessard, L., et al., *Nuclear localization of nuclear factor-kappaB p65 in primary prostate tumors is highly predictive of pelvic lymph node metastases.* Clin Cancer Res, 2006. **12**(19): p. 5741-5.

495. Fradet, V., et al., *Nuclear factor-kappaB nuclear localization is predictive of biochemical recurrence in patients with positive margin prostate cancer.* Clin Cancer Res, 2004. **10**(24): p. 8460-4.

496. Stapleton, A.M., et al., *Assessment of the biologic markers p53, Ki-67, and apoptotic index as predictive indicators of prostate carcinoma recurrence after surgery.* Cancer, 1998. **82**(1): p. 168-75.

497. Wheeler, T.M., et al., *Apoptotic index as a biomarker in prostatic intraepithelial neoplasia (PIN) and prostate cancer.* J Cell Biochem Suppl, 1994. **19**: p. 202-7.

498. Aihara, M., et al., *The frequency of apoptosis correlates with the prognosis of Gleason Grade 3 adenocarcinoma of the prostate.* Cancer, 1995. **75**(2): p. 522-9.

499. Krajewska, M., et al., *Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers.* Am J Pathol, 1996. **148**(5): p. 1567-76.

500. Amirghofran, Z., A. Monabati, and N. Gholijani, *Apoptosis in prostate cancer: bax correlation with stage.* Int J Urol, 2005. **12**(4): p. 340-5.

501. Rubio, J., et al., *Immunohistochemical expression of Ki-67 antigen, cox-2 and Bax/Bcl-2 in prostate cancer; prognostic value in biopsies and radical prostatectomy specimens.* Eur Urol, 2005. **48**(5): p. 745-51.

502. Royuela, M., et al., *IL-2, its receptors, and bcl-2 and bax genes in normal, hyperplastic and carcinomatous human prostates: immunohistochemical comparative analysis.* Growth Factors, 2000. **18**(2): p. 135-46.

503. Chia, S.J., et al., *Prostate tumours from an Asian population: examination of bax, bcl-2, p53 and ras and identification of bax as a prognostic marker.* Br J Cancer, 2000. **83**(6): p. 761-8.

504. Amirghofran, Z., A. Monabati, and N. Gholijani, *Androgen receptor expression in relation to apoptosis and the expression of cell cycle related proteins in prostate cancer.* Pathol Oncol Res, 2004. **10**(1): p. 37-41.

505. Dunsmuir, W.D., et al., *Molecular markers for predicting prostate cancer stage and survival.* BJU Int, 2000. **86**(7): p. 869-78.

506. Li, R., et al., *High level of androgen receptor is associated with aggressive clinicopathologic features and decreased biochemical recurrence-free survival in prostate: cancer patients treated with radical prostatectomy.* Am J Surg Pathol, 2004. **28**(7): p. 928-34.

507. Schafer, W., et al., *Intensity of androgen and epidermal growth factor receptor immunoreactivity in samples of radical prostatectomy as prognostic indicator: correlation with clinical data of long-term observations.* J Urol, 2006. **176**(2): p. 532-7.

508. Theodoropoulos, V.E., et al., *Evaluation of neuroendocrine staining and androgen receptor expression in incidental prostatic adenocarcinoma: prognostic implications.* Urology, 2005. **66**(4): p. 897-902.

509. Tannock, I.F., et al., *Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer.* N Engl J Med, 2004. **351**(15): p. 1502-12.

510. Tolcher, A.W., et al., *A phase II, pharmacokinetic, and biological correlative study of oblimersen sodium and docetaxel in patients with*

hormone-refractory prostate cancer. Clin Cancer Res, 2005. **11**(10): p. 3854-61.

511. Chang, C.Y., et al., *Development of peptide antagonists for the androgen receptor using combinatorial peptide phage display.* Mol Endocrinol, 2005. **19**(10): p. 2478-90.
512. Bander, N.H., et al., *Phase I trial of 177lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer.* J Clin Oncol, 2005. **23**(21): p. 4591-601.
513. Pienta, K.J. and D.C. Smith, *Advances in prostate cancer chemotherapy: a new era begins.* CA Cancer J Clin, 2005. **55**(5): p. 300-18; quiz 323-5.
514. Arora, A. and E.M. Scholar, *Role of tyrosine kinase inhibitors in cancer therapy.* J Pharmacol Exp Ther, 2005. **315**(3): p. 971-9.
515. Loberg, R.D., et al., *Pathogenesis and treatment of prostate cancer bone metastases: targeting the lethal phenotype.* J Clin Oncol, 2005. **23**(32): p. 8232-41.
516. Jain, R.K., *Antiangiogenic therapy for cancer: current and emerging concepts.* Oncology (Williston Park), 2005. **19**(4 Suppl 3): p. 7-16.
517. Chan, S., *Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer.* Br J Cancer, 2004. **91**(8): p. 1420-4.
518. Wu, L., D.C. Birle, and I.F. Tannock, *Effects of the mammalian target of rapamycin inhibitor CCI-779 used alone or with chemotherapy on human prostate cancer cells and xenografts.* Cancer Res, 2005. **65**(7): p. 2825-31.
519. Powers, M.V. and P. Workman, *Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors.* Endocr Relat Cancer, 2006. **13 Suppl 1**: p. S125-35.
520. Heath, E.I., et al., *A phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer.* Clin Prostate Cancer, 2005. **4**(2): p. 138-41.

521. Miyake, H., I. Hara, and M.E. Gleave, *Antisense oligodeoxynucleotide therapy targeting clusterin gene for prostate cancer: Vancouver experience from discovery to clinic.* Int J Urol, 2005. **12**(9): p. 785-94.

522. Zavrski, I., et al., *Molecular and clinical aspects of proteasome inhibition in the treatment of cancer.* Recent Results Cancer Res, 2007. **176**: p. 165-76.

523. Dreicer, R., et al., *Phase I/II study of bortezomib plus docetaxel in patients with advanced androgen-independent prostate cancer.* Clin Cancer Res, 2007. **13**(4): p. 1208-15.

524. Papandreou, C.N., et al., *Phase I trial of the proteasome inhibitor bortezomib in patients with advanced solid tumors with observations in androgen-independent prostate cancer.* J Clin Oncol, 2004. **22**(11): p. 2108-21.

525. Hainsworth, J.D., et al., *Weekly docetaxel and bortezomib as first-line treatment for patients with hormone-refractory prostate cancer: a Minnie Pearl Cancer Research Network phase II trial.* Clin Genitourin Cancer, 2007. **5**(4): p. 278-83.

526. Posternak, S., *Sur la synthèse de l'ether hexaphosphorique de l'inosite avec le principe phhospho-organique de réserve des plantes vertes.* Compt Rend Acad Sci, 1919. **169**: p. 138-140.

527. Vucenik, I. and A.M. Shamsuddin, *Cancer inhibition by inositol hexaphosphate (IP6) and inositol: from laboratory to clinic.* J Nutr, 2003. **133**(11 Suppl 1): p. 3778S-3784S.

528. Deliliers, G.L., et al., *Effect of inositol hexaphosphate (IP(6)) on human normal and leukaemic haematopoietic cells.* Br J Haematol, 2002. **117**(3): p. 577-87.

529. Shamsuddin, A.M., A. Baten, and N.D. Lalwani, *Effects of inositol hexaphosphate on growth and differentiation in K-562 erythroleukemia cell line.* Cancer Lett, 1992. **64**(3): p. 195-202.

530. Sakamoto, K., G. Venkatraman, and A.M. Shamsuddin, *Growth inhibition and differentiation of HT-29 cells in vitro by inositol hexaphosphate (phytic acid).* Carcinogenesis, 1993. **14**(9): p. 1815-9.

531. Yang, G.Y. and A.M. Shamsuddin, *IP6-induced growth inhibition and differentiation of HT-29 human colon cancer cells: involvement of intracellular inositol phosphates.* Anticancer Res, 1995. **15**(6B): p. 2479-87.

532. Arnold, J.T., et al., *Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay.* Cancer Res, 1995. **55**(3): p. 537-43.

533. Vucenik, I., et al., *IP6 in treatment of liver cancer. I. IP6 inhibits growth and reverses transformed phenotype in HepG2 human liver cancer cell line.* Anticancer Res, 1998. **18**(6A): p. 4083-90.

534. Shamsuddin, A.M., G.Y. Yang, and I. Vucenik, *Novel anti-cancer functions of IP6: growth inhibition and differentiation of human mammary cancer cell lines in vitro.* Anticancer Res, 1996. **16**(6A): p. 3287-92.

535. Ferry, S., et al., *Inositol hexakisphosphate blocks tumor cell growth by activating apoptotic machinery as well as by inhibiting the Akt/NFkappaB-mediated cell survival pathway.* Carcinogenesis, 2002. **23**(12): p. 2031-41.

536. Huang, C., et al., *Inositol hexaphosphate inhibits cell transformation and activator protein 1 activation by targeting phosphatidylinositol-3' kinase.* Cancer Res, 1997. **57**(14): p. 2873-8.

537. Zi, X., R.P. Singh, and R. Agarwal, *Impairment of erbB1 receptor and fluid-phase endocytosis and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells.* Carcinogenesis, 2000. **21**(12): p. 2225-35.

538. Shamsuddin, A.M., A.M. Elsayed, and A. Ullah, *Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate.* Carcinogenesis, 1988. **9**(4): p. 577-80.

539. Shamsuddin, A.M., A. Ullah, and A.K. Chakravarthy, *Inositol and inositol hexaphosphate suppress cell proliferation and tumor formation in CD-1 mice.* Carcinogenesis, 1989. **10**(8): p. 1461-3.

540. Shamsuddin, A.M. and A. Ullah, *Inositol hexaphosphate inhibits large intestinal cancer in F344 rats 5 months after induction by azoxymethane.* Carcinogenesis, 1989. **10**(3): p. 625-6.

541. Ullah, A. and A.M. Shamsuddin, *Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats.* Carcinogenesis, 1990. **11**(12): p. 2219-22.

542. Nelson, R.L., et al., *The effect of iron on experimental colorectal carcinogenesis.* Anticancer Res, 1989. **9**(6): p. 1477-82.

543. Shivapurkar, N., et al., *A rapid dual organ rat carcinogenesis bioassay for evaluating the chemoprevention of breast and colon cancer.* Cancer Lett, 1996. **100**(1-2): p. 169-79.

544. Pretlow, T.P., et al., *Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate.* Carcinogenesis, 1992. **13**(9): p. 1509-12.

545. Challa, A., D.R. Rao, and B.S. Reddy, *Interactive suppression of aberrant crypt foci induced by azoxymethane in rat colon by phytic acid and green tea.* Carcinogenesis, 1997. **18**(10): p. 2023-6.

546. Jenab, M. and L.U. Thompson, *Phytic acid in wheat bran affects colon morphology, cell differentiation and apoptosis.* Carcinogenesis, 2000. **21**(8): p. 1547-52.

547. Thompson, L.U. and L. Zhang, *Phytic acid and minerals: effect on early markers of risk for mammary and colon carcinogenesis.* Carcinogenesis, 1991. **12**(11): p. 2041-5.

548. Vucenik, I., Z.S. Zhang, and A.M. Shamsuddin, *IP6 in treatment of liver cancer. II. Intra-tumoral injection of IP6 regresses pre-existing human liver cancer xenotransplanted in nude mice.* Anticancer Res, 1998. **18**(6A): p. 4091-6.

549. Hirose, M., et al., *Modifying effects of the naturally occurring antioxidants gamma-oryzanol, phytic acid, tannic acid and n-tritriacontane-16, 18-dione in a rat wide-spectrum organ carcinogenesis model.* Carcinogenesis, 1991. **12**(10): p. 1917-21.

550. Wattenberg, L.W., J.B. Coccia, and A.R. Galbraith, *Inhibition of carcinogen-induced pulmonary and mammary carcinogenesis by chalcone administered subsequent to carcinogen exposure.* Cancer Lett, 1994. **83**(1-2): p. 165-9.

551. Estensen, R.D. and L.W. Wattenberg, *Studies of chemopreventive effects of myo-inositol on benzo[a]pyrene-induced neoplasia of the lung and forestomach of female A/J mice.* Carcinogenesis, 1993. **14**(9): p. 1975-7.

552. Vucenik, I., G.Y. Yang, and A.M. Shamsuddin, *Comparison of pure inositol hexaphosphate and high-bran diet in the prevention of DMBA-induced rat mammary carcinogenesis.* Nutr Cancer, 1997. **28**(1): p. 7-13.

553. Vucenik, I., et al., *Inhibition of rat mammary carcinogenesis by inositol hexaphosphate (phytic acid). A pilot study.* Cancer Lett, 1993. **75**(2): p. 95-102.

554. Vucenik, I., G.Y. Yang, and A.M. Shamsuddin, *Inositol hexaphosphate and inositol inhibit DMBA-induced rat mammary cancer.* Carcinogenesis, 1995. **16**(5): p. 1055-8.

555. Ishikawa, T., et al., *Inhibition of skin cancer by IP6 in vivo: initiation-promotion model.* Anticancer Res, 1999. **19**(5A): p. 3749-52.

556. Vucenik, I., et al., *Antitumor activity of phytic acid (inositol hexaphosphate) in murine transplanted and metastatic fibrosarcoma, a pilot study.* Cancer Lett, 1992. **65**(1): p. 9-13.

557. Vucenik, I., et al., *Novel anticancer function of inositol hexaphosphate: inhibition of human rhabdomyosarcoma in vitro and in vivo.* Anticancer Res, 1998. **18**(3A): p. 1377-84.

558. Singh, R.P., et al., *In vivo suppression of hormone-refractory prostate cancer growth by inositol hexaphosphate: induction of insulin-like growth*

factor binding protein-3 and inhibition of vascular endothelial growth factor. Clin Cancer Res, 2004. **10**(1 Pt 1): p. 244-50.

559. Irvine, R.F. and M.J. Schell, *Back in the water: the return of the inositol phosphates.* Nat Rev Mol Cell Biol, 2001. **2**(5): p. 327-38.
560. Verbsky, J.W., et al., *The synthesis of inositol hexakisphosphate. Characterization of human inositol 1,3,4,5,6-pentakisphosphate 2-kinase.* J Biol Chem, 2002. **277**(35): p. 31857-62.
561. Berridge, M.J., *Cell signalling. A tale of two messengers.* Nature, 1993. **365**(6445): p. 388-9.
562. Berridge, M.J., *Inositol trisphosphate and calcium signalling.* Nature, 1993. **361**(6410): p. 315-25.
563. Efanov, A.M., S.V. Zaitsev, and P.O. Berggren, *Inositol hexakisphosphate stimulates non-Ca2+-mediated and primes Ca2+-mediated exocytosis of insulin by activation of protein kinase C.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4435-9.
564. Larsson, O., et al., *Inhibition of phosphatases and increased Ca2+ channel activity by inositol hexakisphosphate.* Science, 1997. **278**(5337): p. 471-4.
565. Fukuda, M., et al., *Functional diversity of C2 domains of synaptotagmin family. Mutational analysis of inositol high polyphosphate binding domain.* J Biol Chem, 1995. **270**(44): p. 26523-7.
566. Mehrotra, B., D.G. Myszka, and G.D. Prestwich, *Binding kinetics and ligand specificity for the interactions of the C2B domain of synaptogmin II with inositol polyphosphates and phosphoinositides.* Biochemistry, 2000. **39**(32): p. 9679-86.
567. Voglmaier, S.M., et al., *Inositol hexakisphosphate receptor identified as the clathrin assembly protein AP-2.* Biochem Biophys Res Commun, 1992. **187**(1): p. 158-63.
568. Ye, W., et al., *Inhibition of clathrin assembly by high affinity binding of specific inositol polyphosphates to the synapse-specific clathrin assembly protein AP-3.* J Biol Chem, 1995. **270**(4): p. 1564-8.

569. Norris, F.A., E. Ungewickell, and P.W. Majerus, *Inositol hexakisphosphate binds to clathrin assembly protein 3 (AP-3/AP180) and inhibits clathrin cage assembly in vitro.* J Biol Chem, 1995. **270**(1): p. 214-7.

570. Gaidarov, I., et al., *Arrestin function in G protein-coupled receptor endocytosis requires phosphoinositide binding.* Embo J, 1999. **18**(4): p. 871-81.

571. Solyakov, L., et al., *Regulation of casein kinase-2 (CK2) activity by inositol phosphates.* J Biol Chem, 2004. **279**(42): p. 43403-10.

572. Fisher, S.K., J.E. Novak, and B.W. Agranoff, *Inositol and higher inositol phosphates in neural tissues: homeostasis, metabolism and functional significance.* J Neurochem, 2002. **82**(4): p. 736-54.

573. Hanakahi, L.A., et al., *Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair.* Cell, 2000. **102**(6): p. 721-9.

574. Hanakahi, L.A. and S.C. West, *Specific interaction of IP6 with human Ku70/80, the DNA-binding subunit of DNA-PK.* Embo J, 2002. **21**(8): p. 2038-44.

575. York, J.D., et al., *A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export.* Science, 1999. **285**(5424): p. 96-100.

576. Odom, A.R., et al., *A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control.* Science, 2000. **287**(5460): p. 2026-9.

577. Alcazar-Roman, A.R., et al., *Inositol hexakisphosphate and Gle1 activate the DEAD-box protein Dbp5 for nuclear mRNA export.* Nat Cell Biol, 2006. **8**(7): p. 711-6.

578. Spiers, I.D., et al., *Synthesis and iron binding studies of myo-inositol 1,2,3-trisphosphate and (+/-)-myo-inositol 1,2-bisphosphate, and iron binding studies of all myo-inositol tetrakisphosphates.* Carbohydr Res, 1996. **282**(1): p. 81-99.

579. Graf, E., K.L. Empson, and J.W. Eaton, *Phytic acid. A natural antioxidant.* J Biol Chem, 1987. **262**(24): p. 11647-50.

580. Hawkins, P.T., et al., *Inhibition of iron-catalysed hydroxyl radical formation by inositol polyphosphates: a possible physiological function for myo-inositol hexakisphosphate.* Biochem J, 1993. **294 (Pt 3)**: p. 929-34.

581. Urbano, G., et al., *The role of phytic acid in legumes: antinutrient or beneficial function?* J Physiol Biochem, 2000. **56**(3): p. 283-94.

582. Jariwalla, R.J., *Inositol hexaphosphate (IP6) as an anti-neoplastic and lipid-lowering agent.* Anticancer Res, 1999. **19**(5A): p. 3699-702.

583. Chen, N., W.Y. Ma, and Z. Dong, *Inositol hexaphosphate inhibits ultraviolet B-induced signal transduction.* Mol Carcinog, 2001. **31**(3): p. 139-44.

584. Agarwal, C., et al., *Inositol hexaphosphate inhibits constitutive activation of NF- kappa B in androgen-independent human prostate carcinoma DU145 cells.* Anticancer Res, 2003. **23**(5A): p. 3855-61.

585. Singh, R.P., C. Agarwal, and R. Agarwal, *Inositol hexaphosphate inhibits growth, and induces G1 arrest and apoptotic death of prostate carcinoma DU145 cells: modulation of CDKI-CDK-cyclin and pRb-related protein-E2F complexes.* Carcinogenesis, 2003. **24**(3): p. 555-63.

586. Agarwal, C., et al., *Inositol hexaphosphate inhibits growth and induces G1 arrest and apoptotic death of androgen-dependent human prostate carcinoma LNCaP cells.* Neoplasia, 2004. **6**(5): p. 646-59.

587. Morrison, R.S., et al., *Inositolhexakisphosphate (InsP6): an antagonist of fibroblast growth factor receptor binding and activity.* In Vitro Cell Dev Biol Anim, 1994. **30A**(11): p. 783-9.

588. Baten, A., et al., *Inositol-phosphate-induced enhancement of natural killer cell activity correlates with tumor suppression.* Carcinogenesis, 1989. **10**(9): p. 1595-8.

589. Tantivejkul, K., I. Vucenik, and A.M. Shamsuddin, *Inositol hexaphosphate (IP6) inhibits key events of cancer metastasis: I. In vitro studies of adhesion, migration and invasion of MDA-MB 231 human breast cancer cells.* Anticancer Res, 2003. **23**(5A): p. 3671-9.

590. Sun, S.C., et al., *NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway.* Science, 1993. **259**(5103): p. 1912-5.

591. Westerheide, S.D., et al., *The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition.* Mol Cell Biol, 2001. **21**(24): p. 8428-36.

592. Kasibhatla, S., et al., *DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1.* Mol Cell, 1998. **1**(4): p. 543-51.

593. Inta, I., et al., *Bim and Noxa are candidates to mediate the deleterious effect of the NF-kappa B subunit RelA in cerebral ischemia.* J Neurosci, 2006. **26**(50): p. 12896-903.

594. Sharma, G., R.P. Singh, and R. Agarwal, *Growth inhibitory and apoptotic effects of inositol hexaphosphate in transgenic adenocarcinoma of mouse prostate (TRAMP-C1) cells.* Int J Oncol, 2003. **23**(5): p. 1413-8.

595. Kikkawa, R., et al., *Investigation of a hepatotoxicity screening system in primary cell cultures --"what biomarkers would need to be addressed to estimate toxicity in conventional and new approaches?"* J Toxicol Sci, 2005. **30**(1): p. 61-72.

596. McKeague, A.L., D.J. Wilson, and J. Nelson, *Staurosporine-induced apoptosis and hydrogen peroxide-induced necrosis in two human breast cell lines.* Br J Cancer, 2003. **88**(1): p. 125-31.

597. Berridge, M.V., P.M. Herst, and A.S. Tan, *Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction.* Biotechnol Annu Rev, 2005. **11**: p. 127-52.

598. Argos, P. and M.G. Rossmann, *Structural comparisons of heme binding proteins.* Biochemistry, 1979. **18**(22): p. 4951-60.

599. Ponka, P., *Cell biology of heme.* Am J Med Sci, 1999. **318**(4): p. 241-56.

600. Olson, J.S., *Binding of inositol hexaphosphate to human methemoglobin.* J Biol Chem, 1976. **251**(2): p. 447-58.

601. Litvinov, I.V., et al., *PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor.* Prostate, 2006. **66**(12): p. 1329-38.

602. Lin, Y., et al., *Androgen and its receptor promote Bax-mediated apoptosis.* Mol Cell Biol, 2006. **26**(5): p. 1908-16.

603. Koumakpayi, I.H., et al., *Expression and nuclear localization of ErbB3 in prostate cancer.* Clin Cancer Res, 2006. **12**(9): p. 2730-7.

604. Nelson, W.G., A.M. De Marzo, and W.B. Isaacs, *Prostate cancer.* N Engl J Med, 2003. **349**(4): p. 366-81.

605. Whitaker, H.C., et al., *Androgen receptor is targeted to distinct subcellular compartments in response to different therapeutic antiandrogens.* Clin Cancer Res, 2004. **10**(21): p. 7392-401.

606. Coughlin, S.S. and I.J. Hall, *A review of genetic polymorphisms and prostate cancer risk.* Ann Epidemiol, 2002. **12**(3): p. 182-96.

607. Davis, J.N., et al., *Elevated E2F1 inhibits transcription of the androgen receptor in metastatic hormone-resistant prostate cancer.* Cancer Res, 2006. **66**(24): p. 11897-906.

608. Garabedian, E.M., P.A. Humphrey, and J.I. Gordon, *A transgenic mouse model of metastatic prostate cancer originating from neuroendocrine cells.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15382-7.

609. Greenberg, N.M., et al., *Prostate cancer in a transgenic mouse.* Proc Natl Acad Sci U S A, 1995. **92**(8): p. 3439-43.

610. Kasper, S., et al., *Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer.* Lab Invest, 1998. **78**(6): p. i-xv.

611. Masumori, N., et al., *A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential.* Cancer Res, 2001. **61**(5): p. 2239-49.

612. McDonald, W.H. and J.R. Yates, 3rd, *Shotgun proteomics and biomarker discovery.* Dis Markers, 2002. **18**(2): p. 99-105.

613. Ong, S.E. and M. Mann, *Mass spectrometry-based proteomics turns quantitative.* Nat Chem Biol, 2005. **1**(5): p. 252-62.

614. Crawford, L.J., et al., *Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132.* Cancer Res, 2006. **66**(12): p. 6379-86.

615. Kishida, S., et al., *Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator.* Oncogene, 1997. **15**(24): p. 2899-907.

616. Moskalenko, S., et al., *Ral GTPases regulate exocyst assembly through dual subunit interactions.* J Biol Chem, 2003. **278**(51): p. 51743-8.

617. Ponting, C.P. and D.R. Benjamin, *A novel family of Ras-binding domains.* Trends Biochem Sci, 1996. **21**(11): p. 422-5.

618. Panner, A., et al., *mTOR-independent translational control of the extrinsic cell death pathway by RalA.* Mol Cell Biol, 2006. **26**(20): p. 7345-57.

619. Stojdl, D.F., et al., *VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents.* Cancer Cell, 2003. **4**(4): p. 263-75.

620. Bruce, I.J. and R. Kerry, *The effect of chloramphenicol and cycloheximide on platelet aggregation and protein synthesis.* Biochem Pharmacol, 1987. **36**(11): p. 1769-73.

621. Drake, J.M., C.L. Gabriel, and M.D. Henry, *Assessing tumor growth and distribution in a model of prostate cancer metastasis using bioluminescence imaging.* Clin Exp Metastasis, 2005. **22**(8): p. 674-84.

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An Androgen-Independent Androgen Receptor Function Protects from Inositol Hexakisphosphate Toxicity in the PC3/PC3(AR) Prostate Cancer Cell Lines

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Titre:

Inositol hexakisphosphate and Proteasome Inhibitors Elicit Enhanced Mitochondrial Depolarization in Androgen-Independent Prostate Cancer Cells: Implication of BCL-2 family proteins

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